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GIGAS PROPERTIES AND ACREAGE YIELD IN AUTOTETRAPLOID GALEOPSIS PUBESCENS

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INTRODUCTION.

MORE than ten years ago MÜNTZING (1936) in his survey was able to show that autopolyploids must have played a great rôle in the evolution of plants in nature, though the formation of allopolyploids seems to have been more important (*cf.* DOBZHANSKY, 1947, chapt. VII). Studies on artificially produced polyploids, auto- as well as allopolyploids, have given much information of value for the discussion of the formation and selection of polyploid plants in nature. Most investigations in polyploids have been carried out on plants growing with liberal spacing. In nature, however, new-originated polyploids have to take up competition with other plants, and therefore it is necessary to determine the physiological properties of the new plants grown in closed stands. During the last decades several investigations have been devoted to this problem, but further work is necessary before the discussion of the evolution of polyploid plants can be founded upon a sound experimental basis. The following experiments have been carried out in order to determine the growth of autopolyploid plants grown in closed stands of varied density and to study the morphological, physiological and chemical differences between the polyploid plants and their low-chromosomal originals under the conditions thus realized.

PLANT MATERIAL, EXPERIMENTAL CONDITIONS, AND ANALYSES.

The investigation was made on plants of diploid and autotetraploid *Galeopsis pubescens* ($2n = 16$, $4n = 32$), which were placed at my disposal by Professor A. MÜNTZING of Lund [1]. MÜNTZING's pure line

[1] I beg to proffer my cordial thanks to Professor A. MÜNTZING for the ready courtesy with which he has placed his material at my disposal, and to Professor G. TURESSON for his readiness in permitting me to place the trials in his garden.

»P—A» was used (MÜNTZING, 1937, p. 372, 1941; MÜNTZING and RUNQUIST, 1939). In the early summer of 1941 I received some diploid and tetraploid plants of the line mentioned, and in the autumn of the same year seeds were collected from these plants. The seeds were sown out in 1942 and in 1943 and the seedlings were used for the determinations.

The plants were grown in the botanical garden of the Institute of Plant Systematics and Genetics of the Royal Agricultural College, and the analyses were made at the Institute of Plant Physiology. The experiments comprise two series, A and B, each with materials of both diploid and tetraploid plants. In series A the frequency of plants was 8,16 plants per sq. m., and in series B 16 plants per sq. m. In 1942 the field arrangements were as follows.

Experimental series A.

14.VI The seeds were grown out on humid filter paper in large Petri dishes, and, after five to eight days, the seedlings were planted in soil-boxes.
10.VII The seedlings were planted over into garden beds.
21.VII The plants were transferred to the field. From the tetraploids only those of a normal appearance were used, this to avoid chromosomal aberrants. Plant distance: 35 × 35 cm corresponding to 8,16 plants per sq. m. Size of the plots: 2,5 × 5,0 m = 12,5 sq. m. 98 plants per plot.
25.VIII Flowering began, almost simultaneously for 2*n* and 4*n*.
8.IX First harvest — plants 86 days old.
24.IX Second harvest — plants 102 days old.

Experimental series B.

10.VI The seeds were grown out on humid filter paper in large Petri dishes, and, after five to eight days, the seedlings were planted in soil-boxes.
10.VII The seedlings were planted over into garden beds.
20.VII The plants were transferred to the field. As in series A only normal tetraploids were used. Plant distance: 25 × 25 cm corresponding to 16 plants per sq. m. Size of the plots: 2,5 × 3,5 m = 8,75 sq. m. 140 plants per plot.
25.VIII Flowering began, almost simultaneously for 2*n* and 4*n*.
27.VIII First harvest — plants 78 days old.
18.IX Second harvest — plants 100 days old.

Harvest. — The samples were taken on two consecutive days, the plant-age being calculated on the basis of the first day only. From each plot ten plants were harvested on the first day and another ten on the second day, ten being taken from one half of the plot and ten from the other half. In all cases the harvested plants were surrounded by at least one protective row.

At 8—9 a. m. the plants were taken and brought to the laboratory for an immediate analysis. The plant-size was determined: fresh and dry weight; size of leaves and branches; number of leaves and branches

per plant; leaf-surface per plant. From these values the yield of plant material per ha field-area and per ha leaf-surface was calculated. In order to determine the absorption of mineral substances of the different plants the dried material was analysed according to LUNDEGÅRDH's method of leaf (triple-) analysis (LUNDEGÅRDH, 1938, 1941, 1945).

The intensity of the apparent carbon dioxide assimilation and the respiration of the leaves were in some cases determined according to methods given by LUNDEGÅRDH and BURSTRÖM (1933), BURSTRÖM (1943), and EKDAHL (1944, pp. 10—11). In connection with these experiments the amount of chlorophyll in the leaves was determined and some measurements of the leaf anatomy carried out.

In 1943 the arrangements of the field cultures were the same as those of the previous year. On July 5—7 about five-week-old seedlings were planted out in the field. Samples were taken from 80- as well as 100-day-old plants, but this year only the dry weight of the plants was determined.

The values obtained were as far as possible statistically proved. To enable the results of diploid and tetraploid plants to be compared the relations between their respective values ($4n/2n$) are given in the tables, the statistical significance of the differences between the two races being marked by the following symbols: *** $= P < 0{,}001$, ** $= 0{,}01 > P > 0{,}001$, * $= 0{,}05 > P > 0{,}01$, and 0 $= P > 0{,}05$.

EXPERIMENTAL RESULTS.

Plant size. — Under the conditions given the tetraploid plants were generally coarser than the diploids, but produced smaller amounts of dry matter. At all harvests the tetraploids had larger leaves than the diploids, but the total leaf-surface per plant, as well as per unit field-area, was always smaller for the tetraploids. The branches of the tetraploids were thicker than those of the diploids, but the latter had a greater number of branches per plant and had a larger total branch length. This is evident from Tables 1—5, which furthermore show that the differences between tetraploids and diploids are fundamentally significant.

MÜNTZING (1941) has previously suggested that autotetraploid *Galeopsis pubescens* is more vigorous than its diploid original plants. In the present investigation it is shown that the gigas characteristics are restricted to the morphological structure of the single organs, and

do not comprise the total production of organs and dry matter. On the whole this seems to be a very common result of a reduplication of the chromosome number in plants (PIRSCHLE, 1942 a and b; LEVAN, 1942, 1943 a, 1945).

Growth rate. — It ought to be pointed out that the differences in plant size and growth between the two chromosome races were greatest at the first harvests. This is certainly due to the facts that at first the diploids grow more rapidly and that they are more branched than the tetraploids. While the plants are young they do not occupy much space. The rapid growth and the quick branching of the diploids may thus result in a production of a greater amount of plant material than that produced by the tetraploids. When the plants grew more, the competition in space between them was first noticeable in the diploid plots, and the decrease in growth caused by this competition acted at an earlier date in the diploid plots than in the tetraploid ones. Thus, the differences in acreage yield between the two chromosomal races will be smallest at the latest harvests (Table 1, col. 4). This is also corroborated by the fact that the differences in acreage yield were greatest in those experimental series where the plants were least compactly set (Table 10). In these plots the growth inhibition caused by the competition in space

TABLE 1. *Size of diploid and tetraploid plants of Galeopsis pubescens.*

Age of plants in days	Chromosomal race	Fresh material g per plant	Dry material g per plant	Dry matter g per 100 g fresh material
1	2	3	4	5
A. 8,16 plants per m^2 field-area				
86	2*n*	246	22,4 ± 0,5	9,07
86	4*n*	239	16,8 ± 0,2	7,03
	4*n*/2*n*	0,97	0,75***	
102	2*n*	273	34,6 ± 0,8	12,60
102	4*n*	257	27,7 ± 0,5	10,78
	4*n*/2*n*	0,94	0,80***	
B. 16 plants per m^2 field-area				
78	2*n*	253	12,3 ± 0,3	4,86
78	4*n*	231	10,6 ± 0,2	4,63
	4*n*/2*n*	0,91	0,86***	
100	2*n*	266	25,4 ± 0,6	9,55
100	4*n*	267	22,8 ± 0,4	8,54
	4*n*/2*n*	1,00	0,90**	

TABLE 2. *Growth and morphology of diploid and tetraploid plants of Galeopsis pubescens.*

Number of leaves and branches determined for 20 plants of each chromosomal race.

Age of plants in days	Chromosomal race	Leaf-size cm²	Leaf-surface m² per plant	Number of leaves		Number of branches		Dry weight, % of plant weight		
				per plant	per m²	per plant	per m²	leaves	stalk + branches	flowers
1	2	3	4	5	6	7	8	9	10	11
				A. 8,16 plants per m² field-area						
86	2*n*	2,26	0,152	674 ± 47	5500	78 ± 6	637	28	54	18
86	4*n*	3,15	0,128	406 ± 32	3310	48 ± 3	392	29	53	18
	4*n*/2*n*	1,39	0,84	0,60***		0,62***				
102	2*n*	2,41	0,185	769 ± 93	6210	90 ± 9	735	20	53	27
102	4*n*	3,70	0,168	454 ± 23	3710	54 ± 3	439	24	54	22
	4*n*/2*n*	1,54	0,91	0,59**		0,60***				
				B. 16 plants per m² field-area						
78	2*n*	2,54	0,102	401 ± 25	6420	52 ± 3	832	31	62	7
78	4*n*	2,62	0,077	294 ± 13	4700	38 ± 1	608	30	68	2
	4*n*/2*n*	1,03	0,76	0,73**		0,73***				
100	2*n*	3,09	0,179	579 ± 33	9260	61 ± 3	992	24	54	22
100	4*n*	3,60	0,135	375 ± 26	6000	43 ± 3	688	24	54	22
	4*n*/2*n*	1,16	0,75	0,65***		0,71***				

became effective later on, and the diploid plants had an opportunity to develop a rich ramification on the sparsely grown plot and thus obtained a better start of the tetraploids in this plot than in the densely grown one.

In a preliminary experiment with *Solanum nodiflorum* LARSEN (1943) has also found the same changes in the relation between tetraploids and diploids. A comparison between the results obtained in *Galeopsis* and in *Solanum* will be given in the discussion below.

Leaf-surface. — The size of the assimilating leaf-surface of the plants is partly dependent on the number of leaves per plant and partly on the leaf-size. At all harvests the tetraploids had larger leaves than the diploids, and the differences were in most cases rather great. This is obvious from the mean values given in Table 2, col. 3 and is also evident from the detailed analysis presented in Table 4. In spite of this observation, however, the total leaf-surface of the tetraploids is generally lower than that of the diploids (Table 2, col. 4). This is ex-

TABLE 3. *Length and thickness of branches of diploid and tetraploid plants of Galeopsis pubescens.*

Number and length of branches and length of stalks determined for 20 plants of each chromosomal race. The average thickness of the branches calculated as volume : length.

Age of plants in days	Chromosomal race	Number of branches per plant	Length of branches per plant m	Volume of branches per plant cm³	Average thickness of branches mm²	Length of stalk cm
1	2	3	4	5	6	7
A. 8,16 plants per m² field-area						
86	2*n*	78 ± 6	—	—	—	56 ± 2
86	4*n*	48 ± 3	—	—	—	52 ± 2
	4*n*/2*n*	0,62***				0,93⁰
102	2*n*	90 ± 9	—	—	—	67 ± 2
102	4*n*	54 ± 3	—	—	—	66 ± 2
	4*n*/2*n*	0,60***				0,99⁰
B. 16 plants per m² field-area						
78	2*n*	52 ± 3	8,51	79,5	9,3	54 ± 1
78	4*n*	38 ± 1	4,25	62,5	14,7	52 ± 1
	4*n*/2*n*	0,73***	0,50	0,89	1,58	0,96⁰
100	2*n*	61 ± 3	10,76	106	10,2	72 ± 1
100	4*n*	43 ± 3	6,72	121	18,0	68 ± 1
	4*n*/2*n*	0,71***	0,62	1,14	1,76	0,94**

TABLE 4. *Leaf-size of diploid and tetraploid plants of Galeopsis pubescens.*

The leaves situated at the three lowest nodes of the stalk and the leaves at the lowest four branches are determined, fifty leaves from each chromosomal race. 90-day-old plants of experiment series B with 16 plants per m² field-area.

Chromosomal race	Leaf-size cm²	Leaf-length cm	Leaf-breadth cm	Leaf-length : leaf-breadth	Leaf-thickness mm
2*n*	14,0 ± 0,9	7,0 ± 0,1	3,8 ± 0,1	1,86 ± 0,02	2,5 ± 0,1
4*n*	22,7 ± 2,0	7,2 ± 0,2	5,2 ± 0,1	1,40 ± 0,01	2,9 ± 0,1
4*n*/2*n*	1,62***	1,04⁰	1,36***	0,77***	1,17**

plained by the fact that the diploid plants have a greater number of leaves than the tetraploids (Table 2, col. 5). In comparison with the diploids the tetraploids thus have large but few leaves, and the increase in leaf-size caused by the reduplication of the chromosome number is smaller than the decrease in leaf-number.

The size of leaves in artificially produced polyploids is one of the

qualities most studied, but only a few values have been published of the number of leaves per plant as well as of the total leaf-surface in these plants as compared with their low-chromosomal origins. PIRSCHLE (1942 a) has made one of the most detailed investigations of the sizes of leaves from autotetraploid plants. He found that the induced tetraploids of *Epilobium collinum*, *Antirrhinum majus*, *Tradescantia geniculata*, *Torenia Fournieri*, *Petunia nyctaginiflora*, *Brassica* (»Kohlrabi»), as well as the varieties »eosin» and »weinrot» of *Impatiens balsamina* all had larger leaves than their low-chromosomal origins. The number of leaves per plant, however, was in most cases lower in the tetraploids than in the diploids, the tetraploids of *Torenia* (p. 138) and *Petunia nyctaginiflora* »eosin» (p. 144) only showing small and insignificant differences when compared with their original diploids. Further, LEVAN has shown that induced tetraploids of sugar beet (1942, 1943 a), mangel (1943 a), and red clover (1945) have leaves which are bigger than those of their diploid origins, but that the diploids in all cases have the greater number of leaves per plant. Neither PIRSCHLE nor LEVAN has made any determinations of the total leaf-area per plant, however. LARSEN (1943) found that the leaf-areas (calculated per plant or per unit field-area) of diploid and autotetraploid *Solanum nodiflorum* are »almost equal during the whole period of vegetation» (p. 40; 18 determinations were made during 120 days). In *Solanum gracile*, however, »the proportion between the size of the leaf area of the diploids and the tetraploids changes in the course of the development» (p. 40), a fact that LARSEN also found to apply to the amphidiploids between *Solanum gracile* and *S. insulae-pascalis* on one side, and *S. nigrum* and *S. nitidibaccatum* on the other side. The same fact has been proved by ANDERSSON (1943) for diploid and tetraploid barley grown in the field. GYÖRFFY (1941) has studied the induced polyploids of eight plant species, and he mentions that the leaf-surface was in most of the cases larger in the tetraploids, though sometimes the diploids possessed the greater surface (Table 28 and p. 439). These values seem to refer to the sizes of the leaves only, and not to the leaf-area per plant.

Leaf size and form. — The differences in size and form of diploid and tetraploid leaves have been the subject of a rather thorough analysis (see Table 4). Surface, length, breadth, and thickness have been calculated for all leaves situated at the three lowest nodes of the stalk and at the four basal branches of the first order. It will be seen that the increase in size did not include all dimensions of the leaf. Whereas the lengths are approximately the same in the tetraploids as in the

diploids, the tetraploids have much broader leaves and also thicker ones than the diploids.

Changes of leaf-form generally seem to be the result of a reduplication of the chromosome number in plants. Induced polyploids in most cases have a more or less unchanged leaf-length, whereas their leaves are often highly increased in breadth. Thus, a great many determinations have shown that the length-breadth index is lower in artificially produced polyploids than in the original diploids of several plant species, as has been proved in tomato (JØRGENSEN, 1928; SCHLÖSSER, 1937, 1940), *Petunia* (HESSE, 1938), sugar beet (RASMUSSON and LEVAN, 1939; SCHLÖSSER, 1940), barley (GREIS, 1940), lucerne (JULÉN, 1944), and red clover (LEVAN, 1945). PIRSCHLE (1942 a) found a lower index for six of the eight species, mentioned on page 403, but a higher index for tetraploid *Tradescantia* (p. 136). SCHERZ (1940) found that the tetraploid races of *Vitis* have smaller or larger leaves than their corresponding diploids, but that the length-breadth index is almost the same in all the tetraploids as in the diploids.

Distribution of dry matter. — How is the produced dry matter distributed over the different organs of diploid and tetraploid *Galeopsis* plants? In order to make this clear the weight of some organs has been calculated in per cent of the weight of the whole plant (Table 2, cols. 9—11). These values show that the leaf matter (branch matter, matter of flower organs, respectively) of the tetraploids forms the same percentage part of the total plant weight as that of the diploids, and this is also the case in both series and at all harvests. The diploid and the tetraploid plants began to flower at the same date (see p. 398), but during the first days the diploids developed more flowers than the tetraploids. At the first harvest of series B, two days after the flowering had started, only a few flowers were opened on the tetraploids, whereas on the diploids a great many were to be seen. The weight of the flower organs at this harvest was only two per cent in the tetraploids, but seven per cent in the diploids. Later on, new flowers were developed on the tetraploid as well as on the diploid plants, but on the latter ones the oldest flowers soon fell and then the two races showed a uniform manner of flowering, which is also demonstrated by the weight-values in Table 2. Thus, the determinations show that the *proportions between the quantities of various organs of the two plant races have been very uniform in spite of the great and significant differences in the sizes of plants and organs.*

The same figures have been obtained by PIRSCHLE (1940) in diploid

and in autotetraploid *Petunia nyctaginiflora*. He states that the values of the proportion between the amount of leaves and stalks »stimmen fast siffernmässig überein, gleichgültig ob man auf Frischgewicht (Tabelle 9) oder auf Trockengewicht (Tabelle 10) bezieht» (p. 358). LARSEN (1943) has shown that »the distribution over the individual plant organs of the matter produced was almost the same in 2*n* and 4*n* plants of *Solanum nodiflorum*» (p. 48), and he has also found that this applies to *S. gracile* and *S. nigrum* during the first part of the vegetation period, when no reproductive organs have yet been formed. Later on, however, when the plants begin to flower, and fruits are formed, the »distribution of dry matter in the two types of plant becomes very different» (p. 25), owing to the fact that only a few fruits are reproduced on the rather sterile tetraploid plants.

Induced polyploids are very often more or less sterile, and as in such cases the plants have large and heavy fruits the reproductive organs of the sterile tetraploids represent a smaller part of the plant-weight than those of the diploids. At the same time the leaves form a greater percentage part of the tetraploid plants than of the diploids, and these facts may cause differences in metabolism and in growth between tetraploids and diploids during the later part of the vegetation period. Tetraploid *Galeopsis pubescens* is rather sterile and gives only a few seeds per flower: in 1941 the tetraploids had on an average $0{,}22 \pm 0{,}01$ seed per flower, while the diploids had $2{,}35 \pm 0{,}02$ (4949 flowers from six 4*n*-plants and 2347 flowers from five 2*n*-plants were counted). In spite of this, the weight of the reproductive organs of the tetraploids formed the same percentage part of the whole plant as in the diploids, which must be due to the seeds being small and light. Thus, the results from the experiments with *Galeopsis pubescens* as well as those of LARSEN with tomatoes clearly demonstrate that the consequences of the sterility in induced polyploids as to the distribution of dry matter over various organs of the plants differ according to the sizes and the weights of seeds and fruits.

Changes in size and form of the leaves as the consequence of a reduplication of the chromosome number in plants must have great influences on the physiological function of the leaves (DERMEN, 1940; STÅLFELT, 1943; EKDAHL, 1944). The cellular construction is of a special interest, and has, in some respects, been studied on the material at hand. The comparison between the values of diploids and tetraploids given in Table 5 shows that the tetraploid leaves have epidermal cells with a surface that is, on an average, 1,7 times larger than that of the

TABLE 5. *Size of leaf-cells of diploid and tetraploid plants of Galeopsis pubescens.*

Volume of the epidermal cells calculated as surface-thickness.

	$4n/2n$
Epidermal cells: surface	1,71***
thickness	1,57***
volume	2,69***
Stomatal cells: length	1,39***
Cell number per unit leaf surface: epidermal cells	0,58***
stomatal cells	0,60***
Cell number per leaf: epidermal cells	0,95
stomatal cells	0,97

diploids (the exact figure for the tetraploids is 58, and that for the diploids 100). As, at the same time, the tetraploids have 1,6 times larger leaves than the diploids it is evident that the number of cells per leaf is approximately the same in both races (*viz.* 4*n*—95, 2*n*—100). The stomatal cells of the tetraploid leaves are 1,4 times longer and their number per sq. cm. leaf-area is lower than that of the diploid leaves (*viz.* 4*n*—60, 2*n*—100), but the number of stomata per leaf is found to be almost the same in both types of leaves (*viz.* 4*n*—97, 2*n*—100). The thickness of the tetraploid epidermal cells is about 1,6 times greater than that of the diploid ones. This comparative value is much higher than the corresponding value of the leaf thickness (*viz.* 4*n*—117, 2*n*—100).

Thus, these measurements make it clear that autotetraploid *Galeopsis pubescens* as compared with the corresponding diploid (for the races investigated and under the given experimental conditions) has larger leaves than the latter, and that this increase in size seems to be entirely caused by an increase in cell-size, while the number of cells per leaf has remained unchanged. The tetraploid leaves also have rather large stomata, though the number of stomata per leaf is unchanged.

The changes in the anatomical structure of the leaves just described may cause changes in the physiological function of the leaves. Thus, an increase in the sizes of the epidermal and the stomatal cells, or in the thickness of the leaves, as well as a decrease in the number of stomata per unit leaf-surface, may influence the rapidity of the gas exchange between the leaf-tissue and the surrounding air, and affect the gas and matter transport in the leaf as well. Further, an increase of the sizes of the leaf-cells as well as changes in the relationship of cell volume and cell surface, and variations in the volumes of the cytoplasm and the vacuoles may likewise cause some changes in the func-

TABLE 6. *Apparent CO_2-assimilation of leaves from diploid and tetraploid Galeopsis pubescens.*

Illumination 18000 lux. temperature 22,0 ± 0,2° C, streaming air. The values are averages of three determinations, each three days.

Chromosomal race	Apparent CO_2-assimilation		
	mg CO_2 per hour and g fresh material	mg CO_2 per hour and g dry material	mg CO_2 per hour and 50 cm² leaf-surface
2*n*	1,54	7,90	1,32
	1,51	8,25	1,15
	1,28	6,96	1,24
	Av. 1,44	Av. 7,70	Av. 1,24
4*n*	1,38	8,11	1,46
	1,21	6,72	1,20
	1,09	5,92	1,27
	Av. 1,23	Av. 6,91	Av. 1,31
4*n*/2*n*	0,85	0,90	1,06

TABLE 7. *Respiration of leaves from diploid and tetraploid Galeopsis pubescens.*

Temperature 20,0 ± 0,2° C, streaming air. The values are averages of three determinations, each two days.

Chromosomal race	Respiration		
	mg CO_2 per hour and g fresh material	mg CO_2 per hour and g dry material	mg CO_2 per hour and 50 cm² leaf-surface
2*n*	0,133	0,684	0,0890
	0,138	0,716	0,0819
	Av. 0,136	Av. 0,700	Av. 0,0854
4*n*	0,0986	0,581	0,0870
	0,0984	0,584	0,0816
	Av. 0,0985	Av. 0,583	Av. 0,0843
4*n*/2*n*	0,72	0,83	0,98

tion and the metabolism of the cells (*cf.* DERMEN, 1940; STÅLFELT, 1943; KOSTOFF, 1943; and EKDAHL, 1944). Consequently, the intensity of the carbon dioxide assimilation and the respiration may be changed.

Rates of photosynthesis and respiration. — The apparent carbon dioxide assimilation and the respiration of diploid and tetraploid leaves have been studied in a few experiments. The results given in Tables 6

and 7 show that the tetraploid leaves have a lower rate of photosynthesis than the diploid ones when calculated per unit fresh or dried material. On the other hand, the tetraploid leaves have the greater consumption of carbon dioxide per unit leaf-surface. The respiration is lower in tetraploid than in diploid leaves, whether the values are calculated per unit fresh matter, dried matter or leaf-surface.

Other investigations as to the photosynthesis and the respiration of induced polyploids are summarized in an earlier paper by the author (EKDAHL, 1944, Table 12, p. 26, and Table 14, p. 28). Most of these results agree with those obtained for *Galeopsis pubescens*. Comparisons between physiological properties of diploid and polyploid plants very often give varying values according as the standards of comparison are varied. This is due to the differences in the content of water, sugar or ash substances, and to those differences in the relations between cytoplasmic compounds, cell-wall substances, and volume of vacuoles which are as a rule to be found between the induced polyploids and their low-chromosomal origins (*cf.* DERMEN, 1940, pp. 623—628; EKDAHL, 1944, pp. 26—28, 34—40; NOGGLE, 1946). These differences between diploids and polyploids vary, however, in different plant species and lines with diverse genetical constitution. Thus, it is clear that there must be very complicated differences in the relations between genetical, morphological and physiological properties in induced polyploid plants. This is the reason why the leaves of tetraploid *Solanum nodiflorum* have a respiration rate that is almost 50 % *lower* than that of the diploids, while on the other hand the leaves of tetraploid *Sinapis alba* have a rate that is 50 % *higher* than that of the corresponding diploid leaves (LARSEN, 1943). Further, attention should be paid to the fact that variations found in the relations of physiological properties between diploids and polyploids are as often caused by different reactions of the two plant types as by the variations of such environmental factors as influence the growth or the metabolism of the plants. On this basis the great variation in the comparative values that GYÖRFFY found in his investigations (1941) may probably be explained. These problems seem to be very interesting and further studies of them can certainly be of great importance to our understanding of the nature of polyploid plants, but hitherto very little has been done to this subject.

Chlorophyll content. — The amount of chlorophyll in the leaves is one of the factors that may regulate the photosynthetical rate of the leaves. In Table 8 the relative values from some determinations of the chlorophyll content are given. The tetraploid leaves have a lower chloro-

TABLE 8. *Chlorophyll content of leaves of diploid and tetraploid Galeopsis pubescens.*

Relative values. Averages of three determinations, each three days.

Chromosomal race	Chlorophyll content		
	per g fresh material	per g dry material	per cm² leaf-surface
2*n*	308	150	330
	298	145	358
	306	149	368
	Av. 304	Av. 148	Av. 352
4*n*	325	165	456
	257	120	434
	264	121	479
	Av. 282	Av. 135	Av. 456
4*n*/2*n*	0,93	0,91	1,30

phyll content than the diploids, as calculated per unit fresh or dried matter, but the differences are rather small. The tetraploids have 1,3 times higher chlorophyll content per unit leaf-surface, a value which is considerably higher than the relation between the photosynthetical rate of the tetraploids and that of the diploids.

These results differ somewhat from those obtained by LEVAN (1943 b) with the same line of *Galeopsis pubescens* (P—A) grown at Svalöf. He found that the tetraploid leaves had a lower chlorophyll content than the diploids, even when the unit of leaf-surface was used as a comparison standard. Probably some differences in the prevailing external factors have caused this difference in the results of the two investigations.

Many investigations have been devoted to the chlorophyll content of the leaves of induced polyploids (KOSTOFF, 1938; GYÖRFFY, 1941; LEVAN, 1943 b; PIRSCHLE, 1941, 1942 a; *cf.* also NOGGLE, 1946). As is to be expected, the results of the comparisons between polyploids and their low-chromosomal originals vary, and the variations are probably caused by differences in the plant structure comparable to those discussed on page 408. It is found that the leaves of artificially produced polyploids are very often thicker and consequently darker green than the leaves of their origins. As a rule the polyploids have the higher chlorophyll content when calculated per unit leaf-surface.

The effect of the variations in chlorophyll content on the carbon dioxide assimilation is that of a »weak-light factor» (GABRIELSEN, 1948).

TABLE 9. *The content of mineral substances in leaves, branches and flowers of diploid and tetraploid plants of Galeopsis pubescens.*

Mg atoms of potassium, calcium, nitrogen, and phosphorus in 100 g dry material (105° C). The values are averages of 4—6 samples of each chromosomal race and 2—3 analyses of each sample.

Material	Chromosomal race	Mg atoms in 100 g dry material			
		K	Ca	N	P
A. 8,16 plants per m² field-area. 102-day-old plants					
Leaves	2*n*	68,8 ± 1,4	49,3 ± 1,7	276 ± 5	6,5 ± 0,2
»	4*n*	73,1 ± 0,8	41,6 ± 1,5	293 ± 4	7,6 ± 0,1
	4*n*/2*n*	1,06*	0,84**	1,06*	1,16***
Branches	2*n*	61,1 ± 1,6	30,8 ± 1,6	54,5 ± 1,6	2,3 ± 0,1
»	4*n*	78,7 ± 2,3	33,2 ± 1,7	73,8 ± 0,9	4,3 ± 0,1
	4*n*/2*n*	1,29***	1,08°	1,35***	1,87***
Flowers	2*n*	55,4 ± 1,5	30,2 ± 1,7	191 ± 3	12,2 ± 0,1
»	4*n*	73,8 ± 1,4	31,5 ± 1,7	199 ± 1	14,2 ± 0,1
	4*n*/2*n*	1,33***	1,04°	1,04*	1,16***
B. 16 plants per m² field-area. 100-day-old plants					
Leaves	2*n*	73,2 ± 1,9	50,6 ± 1,5	252 ± 2	5,8 ± 0,1
»	4*n*	74,5 ± 0,8	43,5 ± 0,4	254 ± 1	6,3 ± 0,0
	4*n*/2*n*	1,02°	0,86***	1,01°	1,09***
Branches	2*n*	66,9 ± 1,1	29,1 ± 1,6	54,4 ± 0,4	2,5 ± 0,1
»	4*n*	69,0 ± 1,3	28,2 ± 1,0	59,7 ± 0,2	3,2 ± 0,0
	4*n*/2*n*	1,03°	0,97°	1,10***	1,28***
Flowers	2*n*	53,4 ± 1,6	32,3 ± 1,4	184 ± 1	11,6 ± 0,3
»	4*n*	65,7 ± 1,7	32,7 ± 1,3	187 ± 1	12,7 ± 0,0
	4*n*/2*n*	1,23***	1,01°	1,02*	1,09**

An increase of the chlorophyll content following a reduplication of the chromosome number in plants will thus affect the carbon dioxide assimilation only in those cases in which the chlorophyll content of the original plants is low and limits their rate of photosynthesis.

Content of mineral substances. — It has on several occasions been shown that there are differences in the amount of organic and mineral substances in polyploid plants as compared with the corresponding low-chromosomal plants (*cf.* EKDAHL, 1944; NOGGLE, 1946). For the present material some analyses of the mineral content have been made, and the results obtained are shown in Table 9. Some of the comparative values (4*n*/2*n*) vary rather much, but it is obvious that the tetraploids

contained more phosphorus than the diploids. This difference is statistically significant for the leaves as well as for the branches and the flowers. The nitrogen content shows differences only in the branches, the highest contents being found in the tetraploids, which may be explained by the fact that, physiologically, they were younger than the diploids. Thus, it was observed that the tetraploid plants, at the latest harvests, were still growing while the diploids had stopped growing and begun to mature. Further, it seems probable that the tetraploid flowers contain more potassium, and the tetraploid leaves more calcium, than the diploid ones.

Acreage yield. — Special interest attaches to a comparison between the two chromosomal races as to their acreage yield and its relation to the assimilating leaf-surface. Calculations on these conditions are collocated in Tables 10, 11 and 12. The relative values of the production of dry matter per unit leaf-area (Table 10, col. 7; Table 12) give a good idea of the total growth of the plants in the two experimental series. In both series the diploids have produced a larger total leaf-surface than the tetraploids. The tetraploids of series A (8,16 plants per sq. m.) have produced a smaller amount of dry matter per unit leaf-surface than

TABLE 10. *Acreage yield of dry material of diploid and tetraploid plants of Galeopsis pubescens.*

Kg dry material per ha field-area and per ha leaf-surface, leaf-surface per ha field-area.

Age of the plants in days	Chromosomal race	Dry material, kg per ha field-area				Dry material kg per ha leaf-surface	Leaf-surface, ha per ha field-area
		leaves	branches	flowers	total		
1	2	3	4	5	6	7	8
A. 8,16 plants per m^2 field-area							
86	2*n*	506	980	345	1831	1477	1,24
86	4*n*	400	727	245	1372	1429	1,04
	4*n*/2*n*	0,79	0,79	0,71	0,75	0,90	0,84
102	2*n*	553	1510	759	2822	1870	1,51
102	4*n*	531	1224	506	2261	1651	1,37
	4*n*/2*n*	0,96	0,81	0,67	0,80	0,88	0,91
B. 16 plants per m^2 field-area							
78	2*n*	612	1216	144	1972	1210	1,63
78	4*n*	504	1152	48	1704	1385	1,23
	4*n*/2*n*	0,82	0,95	0,33	0,87	1,14	0,75
100	2*n*	976	2175	912	4063	1416	2,87
100	4*n*	882	1968	800	3650	1690	2,16
	4*n*/2*n*	0,90	0,90	0,88	0,90	1,19	0,75

TABLE 11. *Acreage yield of mineral substances of diploid and tetraploid plants of Galeopsis pubescens.*

Kg dry material, nitrogen and mineral substances per ha field-area.

Material	Chromosomal race	Kg per ha field-area				
		dry material	K	Ca	P	N
A. 8,16 plants per m² field-area. 102-day-old plants						
Leaves	2*n*	553	14,9	10,9	1,11	21,4
»	4*n*	531	15,2	8,9	1,24	21,8
Branches	2*n*	1510	36,1	18,6	1,07	11,5
»	4*n*	1224	37,7	16,3	1,62	12,6
Flowers	2*n*	759	16,4	9,1	2,87	20,3
»	4*n*	506	14,6	6,4	2,23	14,1
Total	2*n*	2822	67,4	38,6	5,05	53,2
»	4*n*	2261	67,5	31,6	5,09	48,5
»	4*n*/2*n*	0,80	1,00	0,82	1,01	0,91
B. 16 plants per m² field-area. 100-day-old plants						
Leaves	2*n*	976	27,9	19,8	1,75	34,4
»	4*n*	882	25,7	15,4	1,72	31,3
Branches	2*n*	2175	56,9	25,4	1,69	16,6
»	4*n*	1968	53,1	22,2	1,95	16,5
Flowers	2*n*	912	19,0	11,8	3,28	23,5
»	4*n*	800	20,6	10,5	3,15	20,9
Total	2*n*	4063	103,8	57,0	6,72	74,5
»	4*n*	3650	99,4	48,1	6,82	68,7
»	4*n*/2*n*	0,90	0,96	0,84	1,01	0,86

TABLE 12. *Size and acreage yield of diploid and tetraploid plants of Galeopsis pubescens. Summer of 1943.*

Age of plants in days	Chromosomal race	Dry material	
		g per plant	kg per ha field-area
A. 8,16 plants per m² field-area			
80	2*n*	24,8	2020
	4*n*	17,9	1460
	4*n*/2*n*	0,72	
100	2*n*	39,1	3190
	4*n*	32,0	2610
	4*n*/2*n*	0,82	
B. 16 plants per m² field-area			
80	2*n*	16,7	2670
	4*n*	13,5	2160
	4*n*/2*n*	0,81	
100	2*n*	27,9	4460
	4*n*	26,1	4180
	4*n*/2*n*	0,94	

the diploids, the reverse being the case in series B (16 plants per sq. m.). This is in agreement with the differences found for the growth inhibition caused by the competition in space, which facts are discussed on page 400.

It is shown that in some cases the tetraploids have a higher mineral content than the diploids, but, in spite of that, the tetraploids never have a higher total content of mineral substances per unit field-area (Table 11).

DISCUSSION.

During the last decades many investigations have been made in order to determine the changes caused by an induced reduplication of the chromosome number in plants. The morphological and genetical constitutions of artificially produced polyploids have been studied as well as the physiological properties and the ecological characters of the plants. The examinations have comprised a great many plant species and crosses; in some cases polyploids from several different genotypes of the same species or crosses have been compared.

All these investigations have brought to light much detailed information about the consequences of induced polyploidy in plants. In spite of this, however, it is at present rather difficult to make any general statement on the subject, and especially to combine the results from different botanical branches. This difficulty is accentuated by the fact that the results of different investigations are often very varying and in some cases contradictory, which is found to be due to variations in the genetical constitution of the original plants used as well as to variations in the cultivating conditions under which the polyploids are studied and compared with their low-chromosomal origins.

Thus, it is found that the original chromosome number is a factor that may determine the results of a reduplication of the chromosome number in plants. Most plant species have an optimum chromosome number, and an increase of this number gives rise to plants with a decreased viability. The number of chromosomes which will give the most vigorous plants varies widely in different species.

The importance of the genotypical variation is demonstrated by the results of the investigations of v. WETTSTEIN *et al.*, who found that the cell enlargement caused by a reduplication of the chromosome number in different genotypes of the moose *Funaria hygrometrica* varied from 1,5 to 8 (*cf.* v. WETTSTEIN, 1940). For the material used in the present investigation, *Galeopsis pubescens*, MÜNTZING (1941, p.

196) has shown that the pollen fertility of the tetraploid race of line P—B is lower than that of line P—A. SCHERZ (1940) and JULÉN (1944) have respectively proved for *Vitis* and *Medicago* that different genotypes give rise to induced polyploids having different leaf-shape. KOSTOFF and AXAMITNAJA (1935), LEVAN (1939), and PIRSCHLE (1940) have compared diploid and autotetraploid plants of *Petunia nyctaginiflora*, and their results show some differences which must be due to differences in the genetical constitution of the original lines used, as well as to variations in the effect of the environmental conditions. Variations in the production of dry matter between different polyploid genotypes have been found by MÜNTZING and by LEVAN (induced polyploids of several crop plants; *cf.* LEVAN, 1945), PIRSCHLE (1942 a and b), FRANDSEN (1945, 1948), and others. The physiological properties of induced polyploids are very variable in the experiments of GYÖRFFY (1941) and PIRSCHLE (1942 a and b).

It is at present evident that the effects of artificially induced polyploidy in plants are very complicated, and it will no doubt take many years before we can elucidate all details of the origin and the development of polyploid plants in nature. The genetical and cytological consequences of a reduplication of the chromosome number in plants are in part well-known (*cf.* DOBZHANSKY, 1947; GUSTAFSSON, 1947), but the physiological and the ecological results are too few to admit of more general conclusions. The results obtained in the autotetraploid line of *Galeopsis pubescens* used in this investigation have only been compared with results from experiments with other induced polyploids, but no character has been mentioned as universally possessed by artificially produced polyploids.

From the present results some calculations can be made which elucidate the effect of a variation of the distance between the plants upon the growth and the acreage yield of diploid and autotetraploid *Galeopsis pubescens*. Each chromosomal race has been cultivated on two plots, one with a density of 8,16 plants per sq. m. (plot A), and another with 16 plants per sq. m. (plot B). For each chromosomal race the differences in plant sizes and in acreage yields between the two plots have been calculated (Table 13). A comparison of the diploid and the tetraploid values gives a good idea of the way in which the different plant types have reacted to a greater plant density or, inversely, how the plants have used the greater space of the plots with the lower plant density. Some of the values will be elucidated below in greater detail.

TABLE 13. *Differences in acreage yield and plant growth between the experimental series A and the series B for diploid and tetraploid plants of Galeopsis pubescens.*

Values from the last harvests of 1942 and 1943.

Determined	Chromosomal race	A 8,16 plants per m² field-area	B 16 plants per m² field-area	Difference B−A total	Difference B−A % of A
September, 1942					
Dry material, kg per ha field-area	2*n*	2822	4063	+ 1241	+ 44
Dry material, kg per ha field-area	4*n*	2261	3650	+ 1389	+ 61
Dry material, kg per ha leaf-surface	2*n*	1870	1416	— 454	— 24
Dry material, kg per ha leaf-surface	4*n*	1651	1690	+ 39	+ 2
Dry material, g per plant	2*n*	34,6	25,4	— 9,2	— 27
» » » » »	4*n*	27,7	22,8	— 4,9	— 18
Leaf-surface, ha per ha field-area	2*n*	1,51	2,87	+ 1,36	+ 90
Leaf-surface, ha per ha field-area	4*n*	1,37	2,16	+ 0,79	+ 58
Leaf-surface, m² per plant	2*n*	0,185	0,179	— 0,006	— 3
» » » »	4*n*	0,168	0,135	— 0,033	— 20
Number of leaves per plant	2*n*	769	579	— 190	— 25
» » » » »	4*n*	454	375	— 79	— 17
Number of leaves per m² field-area	2*n*	6210	9260	+ 3052	+ 49
Number of leaves per m² field-area	4*n*	3710	6000	+ 2290	+ 62
Number of branches per plant	2*n*	90	61	— 29	— 32
Number of branches per plant	4*n*	54	43	— 11	— 20
Number of branches per m² field-area	2*n*	735	992	+ 257	+ 35
Number of branches per m² field-area	4*n*	439	688	+ 249	+ 57
September, 1943					
Dry material, kg per ha field-area	2*n*	3190	4460	+ 1270	+ 40
Dry material, kg per ha field-area	4*n*	2610	4180	+ 1570	+ 60

The production of dry matter per ha field-area was largest on the more densely grown plots as a result of the higher number of plants per area unit. Plots B had about twice as many plants per sq. m. as had plots A, but the diploid plants grown on B produced only 44 % more dry matter than those on plot A. The tetraploid plants of plot B showed a corresponding increase of 61 %. These values apply to the year 1942, and the next year the corresponding values were 40 % for the diploids and 60 % for the tetraploids. It is obvious that the diploid plants are able to use the greater space of the low-density plots better than the tetraploids (*cf.* p. 400). Expressed in another way: on the high-density plots the tetraploids are not so depressed in growth by the space competition as the diploids.

The formation of total leaf-surface per ha field-area has increased with the increase of the plant density. In the diploid plants there has been an increase in almost the same degree as in the number of plants per plot, while the tetraploids have produced a total leaf-surface that was increased only about half as much.

The values from 1942 admit of some calculations respecting the *relation between the production of dry matter and the total leaf-area*, or the net assimilation per unit leaf-surface, and comparisons between diploid and tetraploid values from plots with different density of the plants. The total leaf-area of the diploids has been about 90 % higher in plot B than in plot A, but the dry matter production was increased only by 44 %. For the tetraploids the total leaf-area has been only 58 % higher in plot B than in plot A, and furthermore the dry matter production was increased to nearly the same degree, or by 61 %. Thus, the production of dry matter per unit leaf-area is 24 % lower in the low-density diploid plot than in the corresponding high-density plot. This must be due to the fact that the total leaf-area has been increased to such a degree that the leaves have shaded each other to a great extent. Between the two tetraploid plots, however, there is almost no difference, which means that the dry matter production of the tetraploids has been proportional to the leaf-area.

In a preliminary experiment LARSEN (1943, p. 20) determined the effect of a variation of the distance between diploid and autotetraploid *Solanum nodiflorum*. The results are not quite significant, but they show that the differences between the diploids and the tetraploids are probably smallest when the density of the plants is greatest. This agrees very well with the results obtained from the more detailed investigations of *Galeopsis pubescens* given in this paper. LARSEN found that when

grown with a density of 1,6 plants per sq. m. tetraploid *Solanum* plants produced in 103 days about 20 % less dry matter than the corresponding diploids. Grown with a density of 44,5 plants per sq. m., however, the tetraploids yielded 7 % more than the diploids. Increase of the plant density thus caused an inversion of the relation between the tetraploid and the diploid *Solanum* plants. It may be supposed that a plant density can be found, higher than that used for plots B in the present investigation, at which tetraploid plants of *Galeopsis pubescens* produce as much dry matter as diploid ones; at a very high plant density the tetraploids may perhaps produce more dry matter than the diploids.

Knowledge of this fact and of the consequences of the morphological characteristics of the plants may be a good help in further studies of polyploid plants as well as for our understanding of the development and the origin of polyploids in nature.

Since the colchicine method made it easy to produce induced polyploids of most plant species many artificially produced polyploids of crop plants have been tested in plant breeding. During the first years great expectations were had of the gigas characters of polyploid plants, and the new plant material was expected rapidly to give rise to high-producing races. The sterility and the slow growth-rate of many induced polyploids, however, have offered great difficulties to plant breeders, which can only be overcome by extensive combination and selection experiments.

Some of the results of the present investigation can be applied to the breeding of induced polyploids. Thus, it may be concluded that *the polyploids in some cases give their highest yield under other cultivating conditions than the diploids.* If it is desired to compare induced polyploids with common diploid races, the comparisons must be made with plants grown in the field under ordinary conditions as well as under various other conditions in order to find out where the polyploid plants give the highest yield. The distance between the plants (the rows), the sowing depth, the date of sowing, the date of late fertilizing, and the date of harvest should thus be varied. In breeding of induced polyploids the best results will certainly be reached when as many polyploid genotypes as possible are compared with their low-chromosomal origins under as varying environmental conditions as possible.

Experiments made with induced polyploids have hitherto shown variable results, and it is difficult to get a general scheme of the changes following a reduplication of the chromosome number in plants. It is, however, this very variation that makes artificially produced polyploids

such a valuable material in plant breeding, and, in the same way, the manifold of possibilities to new gene combinations has probably been one of the causes of the wide distribution of polyploids in nature.

SUMMARY.

This investigation has been carried out in order to determine the morphological characters and the physiological properties as well as the growth and the acreage yield of an induced autopolyploid plant, and to compare the results obtained with the corresponding values of the original diploid plant. Diploid and autotetraploid *Galeopsis pubescens* have been used (MÜNTZING's pure line »P—A»; MÜNTZING, 1937, p. 372, 1941), and the plants have been grown in the field in two series with different plant density.

Under the given conditions the tetraploids are generally coarser than the diploids, but they produce smaller amounts of dry matter and a lower number of leaves and branches. The gigas characteristics of the tetraploids are restricted to the morphological structure of the single organs and do not comprise the total production of organs and dry matter.

The differences in plant size and in growth between the two chromosomal races are greatest at the earliest harvest. The inhibition of the growth caused by competition in space has been most pronounced in the diploid plot with the highest plant density.

The tetraploids have larger leaves than the diploids but a lower number of leaves per plant. The increase in leaf-size is smaller than the decrease in leaf-number, and thus the diploid plants have the largest total leaf-area per plant.

The length-breadth index of the leaves is lowest for the tetraploids.

The proportions between the quantities of various organs of the two plant-races are very uniform in spite of the great and significant differences in the sizes of plants and organs.

The increase in the size of the tetraploid leaves seems to be entirely caused by an increase in the cell-size, while the number of cells per leaf has remained unchanged.

The tetraploid leaves have a lower photosynthetical rate than the diploid ones with the unit fresh or dried matter as a standard, but a higher rate per unit leaf-area. The respiration is lowest for the tetraploid leaves.

The tetraploid leaves have a lower chlorophyll content than the

diploids with the unit fresh or dried matter as a standard, but a higher chlorophyll content per unit leaf-area.

The diploids have the greatest acreage yields. The difference between diploids and tetraploids is greater at the first harvest than at the later, and greater between the plots with the lowest plant density than between the more densely grown plots.

The diploids seem to be able to use the greater space of the low-density plots better than the tetraploids, but the tetraploids are not so depressed in growth by the space competition in the high-density plots as the diploids.

The production of dry matter per unit leaf-area is lower in the low-density diploid plot than in the corresponding high-density plot, but in the tetraploid plots with different density of the plants the dry-matter production is proportional to the leaf-area.

It is concluded that induced polyploids in some cases give their highest yield under other cultivating conditions than their low-chromosomal origins.

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GENETICS, ORIGIN AND EVOLUTION OF KINETOCHORES

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THE term kinetochore (= movement place; SHARP, 1934) is used in this paper to mean the specialized region of the chromosome where the property of active mobility on the spindle is manifested irrespective of the number or extension of these regions in the chromosome body and of other properties that may be associated with the region of active mobility.

The expression localized kinetochore is used when a single region is present in the chromosome body which manifests the property of active mobility in conjunction with the property of a special cycle of division.

The expressions diffuse and multiple kinetochores, respectively, are used to mean the apparent distribution of the property of active mobility throughout the whole body of the chromosome, and the existence of several distinct regions in the chromosome body that have this property. The property of a special cycle of division cannot always be recognized in the first of these types. The term non-localized kinetochore is used when the type of kinetochore found is not a localized one, but when it may or may not be of the diffuse type.

The term centromere is used as a synonym of localized kinetochore because it has almost acquired this meaning in the literature. Besides, the term centromere (understood as a region which is the centre of the forces responsible for the chromosome behaviour during division) is mainly a term having a genetic significance. Thus, the crossing-over per unit cytological length near the centromere is lower than elsewhere. The term centromere lacks the universality necessary to embrace all the regions in question. These regions have in common only one property, the property of active mobility, and among the existing terms, kinetochore — movement place — is the one which expresses best this phenomenon. It has the advantage that it does not imply the existence of any specialized »organ» or »body» at this region of the chromosome, and it is independent of the structure of the region in question. I do not, however, consider it an ideal term, but, as it is fairly good for the exposition

of the present problem, it may be used until more is known about this phenomenon.

I. THE GENETICS OF THE LOCALIZED KINETOCHORE.

Recently the writer (LIMA-DE-FARIA, 1949) has observed at pachytene the existence of centromeric chromomeres in the chromosomes of rye. According to these observations, the centromere is composed of chromomeres quite similar in appearance to those existing in the arms of the chromosomes, and the fibrillae connecting the centromeric chromomeres are also similar in appearance to those connecting the chromomeres of the arms. Hence the rye chromosomes have shown that there is no *essential* difference in a morphological respect between the structure of the centromere and the structure of the arms. Both are composed of fibrillae and chromomeres.

This does not necessarily imply that the centromere may have the same submicroscopic structure or chemical constitution as the chromosome arms (or perhaps more precisely, as the euchromatin of the chromosome arms). To a certain extent it must have a different chemical constitution. The fact remains, however, that when considered on a microscopic scale it does not seem to be essentially different from the chromosome arms but only as a specialized region of the chromosome body.

In the pairs of rye chromosomes observed at pachytene it is possible to recognize three distinct zones in the centromere, *viz.* (1) A first zone, the exterior zone, in which the two fibrillae are almost unstained. This exterior zone forms on both sides of the centromere, the connection with the chromomeres of the arms. (2) A second zone, the chromomeric zone, composed of two pairs of centromeric chromomeres. These two pairs of chromomeres are disposed in longitudinal sequence and each pair is attached to the two almost unstained fibrillae that compose the exterior zone. (3) A third zone, the interior zone, comprises the space between the two pairs of centromeric chromomeres; two slightly stained fibrillae can be observed in it. This zone is characterized by a deeper staining than the first zone. There are reasons (see LIMA-DE-FARIA, *l. c.*) to believe that this zone is composed of chromomeres of smaller size. If we start from one end of the centromere, considering the two paired chromosomes, we can see the following sequence: two almost unstained fibrillae, a pair of chromomeres, two slightly stained fibrillae (which

may contain chromomeres of smaller size), another pair of chromomeres and again two almost unstained fibrillae.

If we imagine a plane passing through the middle of the centromere the latter becomes divided into two parts related one to the other as an object to its image in a plane mirror. It has a symmetric structure.

The two halves of the centromere are not only morphologically similar but there is evidence that they are also genetically alike. This evidence rests on two observations. MÜNTZING (1944) has shown — on the basis of size and pairing behaviour — that the »standard fragment» found in rye (a type of B chromosome), which possesses one short and one long arm, has given rise to two iso-chromosomes presumably by misdivision of the centromere, one iso-chromosome resulting from the doubling of the short arm (small iso-fragment) and the other from the doubling of the long arm (large iso-fragment). The centromere of the standard fragment had misdivided and both halves had given origin by doubling to two new chromosomes which both had equal ability to perform division movements in subsequent generations of cells. This observation suggests that both halves of the centromere have equal potentiality, each giving rise to new centromeres having the same characteristics. Confirmation of the opinion that the large iso-fragment is derived from the doubling of the long arm of the standard fragment has been obtained by an analysis of the structure of these two types of chromosomes at pachytene (MÜNTZING and LIMA-DE-FARIA, 1949).

The second source of evidence was obtained (MÜNTZING and LIMA-DE-FARIA, *l. c.*) from the pairing behaviour of the large iso-fragment at pachytene. In those cells in which a large iso-fragment does not pair with another large iso-fragment the two identical arms of the large iso-fragment unite and pair with each other throughout their entire length. In this case the two halves of the centromere have also been seen to be paired with one another. This may or may not be a mechanical consequence of the pairing of the two arms. This fact is still another indication of the genetic similarity (if not identity) of both halves of the centromere.

For these reasons the centromere of the chromosomes of rye may be called a *reversed repeat.*

All these observations show the existence of several points of similarity, on a microscopic scale, between the centromere and the arms of the chromosome.

Experimental evidence suggesting the existence of genes at the centromere has also been reported.

AUERBACH (1947), after treatment of wild type males of *Drosophila melanogaster* with a mustard gas compound, observed an abnormal and inherited type of segregation, which points to an effect of the chemical on the centromere of the treated *X* chromosome.

The treated *X* chromosome was characterized by four peculiarities: it carried a semilethal, it carried a sterility mutation, it had a tendency to follow the untreated *X* chromosome into the same cell at meiosis (non-disjunction), and it had a still greater tendency to be lost on the spindle at meiosis. According to AUERBACH, the viability and the sterility mutation were possibly identical, having been located in the same region as carried the cause of the abnormal segregation, for all peculiarities were lost simultaneously from one female. This region appears to have been the centromere region. She considers that under the influence of the chemical treatment a change took place in the centromere region, either a small rearrangement involving the centromere, or a »mutation» of the centromere itself. The examination of salivary gland slides revealed an abnormality in the region of the centromere, but its nature could not be determined.

There has not been found until now, as far as I know, any other experimental evidence supporting the idea of the existence of genes at the centromere, but nor do we know to what extent such a fact depends on the present methods employed. Mustard gas compounds, when compared with X-rays, have been found to have a somewhat different action on the chromosomes.

II. THE ORIGIN OF NEW KINETOCHORES.

PRAKKEN and MÜNTZING (1942) have studied the property of active mobility exhibited by the ends of some chromosomes of rye, simulating a terminal centromere. The chromosomes of rye have a median or submedian centromere, but in some inbred lines the authors found that a chromosome pair seemed also to have a terminal centromere. The ends of the short arms of these chromosomes were stretched and turned to the poles. A detailed study led these workers to the conclusion that this »T-chromosome» pair (»terminal» chromosomes; a term used by KATTERMANN) did not carry a complete centromere similar to the localized submedian kinetochore, but that these »T-ends» were only similar to the localized kinetochore in some aspects. By their work and by the work of ÖSTERGREN and PRAKKEN (1946), carried out with the same slides restained by a different technique, it could be established

that T-ends and localized kinetochores had the following similarities and differences, *viz.*

(1) Similarities:

(a) Evidence of attraction between spindle poles and T-ends. T-ends and centromeres had the same mechanism of movement on the spindle.

(b) Chromosomal fibres were also attached to T-ends.

(c) The T-ends were found at anaphase I to move at least as rapidly as centromeres.

(d) Evidence of the existence of an inheritable difference between a T-chromosome and its homologue in normal lines was obtained from the study of meiosis in the F_1 hybrids resulting from a cross between lines with and without a T-chromosome pair.

(2) Differences:

(a) The property of a special cycle of division was lacking in the T-ends. At anaphase I four separate T-ends could be observed in most cells, and at interphase there was not a terminal centromere connecting the two chromatids.

(b) The T-phenomenon was only observed at meiosis.

(c) The characteristic features of the T-chromosomes were expressed to different degrees in different lines. The various meiotic stages were not equally responsive to differences in the intensity of the T-phenomenon.

(d) In one inbred line more than one pair of chromosomes were involved, from 0 to 6 pairs of chromosomes showed the T-phenomenon more or less clearly.

From these observations it can be seen that we are not faced with an instance of a localized kinetochore, but that the T-ends have in full (at meiosis) one of the properties of localized kinetochores, active mobility, but that they completely lack the second property of the centromere, a special cycle of division. Moreover, they are affected by the genotypical constitution of the line by which they are carried and by the different physiological conditions found in the same individual. In some organisms the diffuse kinetochore shows a striking similarity in its properties with the T-ends of rye. The diffuse kinetochore displays the property of active mobility, is affected by physiological conditions, and

does not, so far as can be detected, possess the property of a special cycle of division.

KATTERMANN (1939) described a quite similar case in another variety of rye, but he did not study it so carefully as the authors cited and was led to the conclusion that the T-phenomenon originated as a result of a translocation between the T-chromosome and an ordinary chromosome with a submedian centromere, the resulting T-chromosome having two localized kinetochores or parts of localized kinetochores. KATTERMANN's interpretation does not meet the facts: firstly, the T-ends have not all the properties of complete centromeres, and for this reason his first assumption ought to be rejected, the second one, *viz.* that they are parts of centromeres is very improbable, because neither KATTERMANN nor PRAKKEN and MÜNTZING, nor ÖSTERGREN and PRAKKEN have seen any abnormal behaviour in the active mobility of the median and submedian centromeres of the ordinary chromosomes, which would have been a most likely result of the lack of the translocated centromeric segment. Besides, the existence in one case of 6 pairs of chromosomes showing the T-phenomenon makes the assumption of a translocation extremely improbable.

The origin of the T-ends is better understood from two observations made by the same authors (PRAKKEN and MÜNTZING, *l. c.*), seen in the light of our present knowledge of the structure of the centromere of the chromosomes of rye. The first observation was that there exists an inheritable difference between the T-chromosome pair and the ordinary chromosomes. The second was that it is the short arm of the T-chromosome which shows the T-phenomenon, and it also seemed to be the shorter arms which were involved in the line with a general T-tendency. According to LEVAN (1942), these ends of the short arms are heterochromatic in five pairs of chromosomes. This observation of LEVAN's has been confirmed by LIMA-DE-FARIA (unpublished). At pachytene, I have observed that at least five of the seven chromosomes of the genome of rye possess a terminal knob in the short arm.

The main result of all these observations is that the existence of chromosome ends having active mobility is accompanied by an inheritable difference of the chromosome pair involved and by the presence of heterochromatin.

The coincidence of these two facts suggests that the T-phenomenon is the result of a hereditary change in the heterochromatic end of the short arm, which has led to a formation possessing one of the properties

of the localized kinetochore and the property common to all kinetochores.

Of a striking similarity to the T-ends of rye are the »secondary centric regions» found in maize by RHOADES and VILKOMERSON (1942). Here, also, the point of secondary active mobility of the chromosome is localized at the distal portion, and is also connected with the spindle. As in rye, the »secondary centric regions» (as the authors call them) may be formed by one or more of the four arms comprising each dyad, and the phenomenon is only limited to the two meiotic divisions. These »secondary centric regions», which moved poleward still more rapidly than the primary centric regions, appeared only in plants of maize having an abnormal type of chromosome ten with extra chromatin near the distal end of the long arm. Plants homozygous for this abnormal tenth chromosome showed a high frequency of »secondary centric regions», while plants heterozygous for it showed them in less quantity. RHOADES (in the press; cited by SCHRADER, 1947) has further found that in the absence of the localized kinetochore there is no indication of a functioning »secondary centric region». It is a pity that the paper of RHOADES has not yet, as far as I know, been published, and as the present information is scanty I will not discuss this detail (however, the existence of a position effect may be suspected).

These observations show, for the same reasons as have reference to rye, that there is no evidence supporting the idea of any translocated centromere or translocated portion of a localized kinetochore. On the contrary, in this case, the connection of the T-phenomenon with a hereditary change not involving the centromere is still clearer owing to the abnormal type of chromosome having had extra chromatin near the distal end of the long arm.

It is a well established fact that dicentric fragments — with two ready-made centromeres — are unable to perform regular division, being broken at anaphase and giving origin to two distinct fragments each possessing a centromere. This seems to be an insuperable obstacle to the maintenance on the chromosome of a secondary particle possessing active mobility, though actually it is not so. In reality events take another course.

An observation made by RHOADES and VILKOMERSON (*l. c.*) is of particular significance in this connection. In both meiotic divisions a »regular disjunction» usually occurred and these plants showing the »secondary centric region» were »very fertile». This fact shows that the existence of a secondary particle with the property of active mobility

existing in chromosomes with a localized kinetochore does not always lead to abnormal behaviour at division, and indicates that a process of adaptation has taken place which ensures regular disjunction and fertility. The T-phenomenon was only observed at the two divisions of meiosis, but the possibility cannot be excluded that the same mechanism of adaptation would be able to operate during mitosis.

In both rye and maize it was the chromosome ends that showed the T-phenomenon. It is well known that in many organisms and during certain stages the ends of chromosomes may show polarization and mobility.

A special activity of chromosome ends has been well established by HUGHES-SCHRADER (1943). During the meiotic prophase of the males of several species of mantids the ends of the chromosomes are involved in an active mobility towards the division centres, which results in the so-called bouquet stage. As HUGHES-SCHRADER points out, the bouquet stage is not a peculiarity of the meiotic prophase of male mantids but is a characteristic feature of meiosis in many animals and some plant species.

The resemblance and mutual attraction between localized kinetochores and chromosome ends observed by RIBBANDS (1941) in his work on the meiosis of Diptera add strength to the evidence that there is a structural similarity between chromosome ends and centromeric regions. Ends of chromosomes exhibit the properties of incipient kinetochores.

The centromeric property exhibited by the ends of certain chromosomes of rye and maize is only the property of active mobility.

Even if this phenomenon helps us very much to understand the origin of the essential property common to all kinetochores and the only property detectable in most of the diffuse kinetochores, it is not sufficient by itself to explain the origin of the localized kinetochore in which the property of a special cycle of division has become intimately associated with the region of active mobility.

Zones of chromosomes showing this second property of the centromere have also been found, but at the time they were observed their nature could not be determined.

They are found in certain types of B chromosomes of a number of species, and are the cause of non-disjunction at anaphase of the first pollen division. These zones with a special cycle of division have been well established by cytological observation in rye (MÜNTZING, 1946 and HÅKANSSON, 1948), in *Anthoxanthum* (ÖSTERGREN, 1947 b) and in

Festuca (BOSEMARK, personal communication). They are observed only at the first pollen mitosis, and in rye also at the corresponding stage on the female side. At this pollen division, when anaphase begins, the B chromosome generally lags between the anaphase groups divided into two chromatids, which cannot separate because they are held together at two zones of the chromosome; finally, as the two chromatids are firmly attached at these two regions, they pass united to the anaphase group which will later become the generative nucleus. In the proceeding division the coherence of the chromatids is not manifested and the B chromosomes divide and separate like the other chromosomes.

At the time MÜNTZING (1946) and ÖSTERGREN (1947 b) published their results it was not possible to decide whether this phenomenon was due (1) to a delayed reproduction or (2) to some kind of chromatid attraction or (3) localized stickiness at these zones. Presently, MÜNTZING and LIMA-DE-FARIA (*l. c.*) observed these types of B chromosomes of rye at pachytene, and the appearance of the chromomeres at these regions does not support the idea of the existence of any special segment with a particular morphology, which could be responsible for stickiness at this zone. For this reason only the first two possibilities are left, and both may be responsible for what can be called a special cycle of division — the other property of the localized kinetochore. The fact is that we do not know at present whether the property of a special cycle of division of the localized kinetochore is the result of a delay in reproduction or the result of chromatid attraction, which are the two causes assumed above to be responsible for the peculiar behaviour of the zones mentioned.

It may be noted that in two of these three cited species the B chromosomes are heterochromatic, a common feature of B chromosomes.

It may be assumed that this property could become permanent, manifesting itself through all the division cycle of the plant. This transformation would lead to a non-disjunction of the B chromosomes at each cell generation, which would have disastrous consequences, although, if this same region, with a special cycle of division, should get the same cycle as the centromere, no obvious trouble would occur.

Summing up, it should be observed that differentiated parts of chromosomes possessing separately the two properties of localized kinetochores arise under normal conditions, that there is no evidence supporting the idea that they receive their origin from the centromeres already existing, and that they seem to be more easily formed in regions which are heterochromatic.

It has been seen that the two properties of localized kinetochores may be exhibited independently by different parts of chromosomes without having a »negative» effect on the behaviour of these chromosomes. If the two properties in question were to occur one after the other in the same region, it may now be seen to what extent the new centromere would be able to survive.

SCHRADER (1939) and ÖSTERGREN (1947 a) have pointed out that, owing to the fact that the actively mobile component of the centromere is divided at metaphase and already at prophase, the property possessed by the centromere of remaining undivided until the end of metaphase of mitotis and until the second division of meiosis must be carried by a material part that is not the mobile component. In a review of the question (LIMA-DE-FARIA, *l. c.*) I have come to the conclusion that the property of active mobility may be attributed to the two chromomeres found in the centromere of each chromosome of rye and that the property of a special cycle of division could be attributed to the slightly stained fibrilla existing between these two chromomeres. In any case, it is evident from the work of SCHRADER and ÖSTERGREN that the two properties must be carried by two different material components.

For this reason the chance of a simultaneous occurrence of the two properties seems to be extremely low. On the other hand, their occurrence one after the other in the same place would not seem to be extremely rare, this being due to the fact that segments having both properties of localized kinetochores are known to occur in the same organism. They seem to occur with a certain frequency; KATTERMANN as well as PRAKKEN and MÜNTZING found the T-phenomenon in two different varieties of rye (inbred material).

If a new centromere is formed by the process outlined above a dicentric chromosome will arise, and the case does not offer difficulties; in the subsequent cell generations the dicentric chromatids will lie between the poles at anaphase, and will break, giving rise to two distinct fragments each carrying a centromere. It may also happen that a polycentric chromosome is formed (see below).

COLEMAN (1948) observed in the chromosomes of some Western species of *Trimerotropis* a centromere shift from an acrocentric to a metacentric position. Two of the species showed both homo- and heterozygosity for the shift. COLEMAN could not find any evidence — from the pairing of the chromosomes at the pachyphase stages — of a pericentric inversion or of an intrachromosomal translocation. For this reason he was led to consider that »the possibility that the apparent

shift is due to the disappearance of the original subterminal centromere and the organization of a new one in a submedian position, while it seems improbable, should not be entirely excluded».

III. THE EVOLUTION OF THE KINETOCHORE.

The work of the SCHRADERS and their collaborators carried on during the last 15—20 years has been a decisive contribution to the establishment of the existence of a non-localized kinetochore in many organisms.

HUGHES-SCHRADER and RIS (1941) have for the first time demonstrated, on an experimental basis, the diffuse nature of the kinetochore in coccids. Males and females of *Steatococcus* were X-rayed: the chromosome fragments obtained behave mitotically like unbroken chromosomes. Each fragment, irrespective of size, oriented typically at metaphase, produced its own half spindle, and the parallel halves disjoined, without lagging at anaphase, like complete chromosomes. This normal behaviour at division permitted the fragments to perpetuate themselves mitotically through many cell generations. The fact that the fragments irrespective of their size and number behave normally in mitosis like complete chromosomes, combined with the observation of an apparent production of half-spindle fibres through the entire length of the chromosomes and of the fragments, is definite evidence that the chromosomes of *Steatococcus* have a diffuse kinetochore spread over the whole body of the chromosome.

The diffuseness of the kinetochore was also experimentally confirmed by RIS (1942) in Aphids. By X-raying parthenogenetic females he obtained mitotic divisions with fragmented chromosomes; these fragments were connected with the poles by chromosomal fibres, oriented in the metaphase plate like unbroken chromosomes and underwent the anaphasic movements regularly.

On evidence from another source HUGHES-SCHRADER (1942) has been able to demonstrate the diffuse nature of the kinetochore in *Nautococcus schraderae* because there is a marked precocity in the formation of half-spindle components relative to the contraction of the meiotic chromosomes. The half-spindle fibres form while the chromosomes are still in late diakinesis, and for this reason she has been able to demonstrate that they arise from a longitudinal zone coextensive with the entire length of the chromosome.

Over and beyond the cases in which the existence of the diffuse

kinetochore has been demonstrated there are many organisms in which the existence of a diffuse or a non-localized kinetochore has been observed or suggested.

Among insects, the first cases reported in detail of the observation of a non-localized kinetochore are those of SCHRADER (1931) in *Protortonia primitiva* and HUGHES-SCHRADER (1931) in the primitive coccid *Llaveia bouvari*. SCHRADER (1935) observed the same phenomenon later in the *X* chromosome of *Protenor belfragei*, and HUGHES-SCHRADER (1940) in the male of *Llaveiella taenechina*.

Parallel anaphase disjunction of chromosomes has also been reported by several authors: in *Gerris* by GEITLER (1938), in *Pediculoides ventricosus* by PÄTAU (1936), who, in the anaphases of the cleavage and blastomere divisions, observed that the chromosomes had »lateralen Spindelansatz», in *Pediculopsis graminum* by COOPER (1939) in which the chromosomes undergo parallel displacement during anaphase in the cleavage divisions of the egg, and as he says — at metaphase — »each chromosome may appear to possess an individual sheaf-like spindle».

Not only in the Hemiptera and in the Acari but also in the whole group of the Lepidoptera a non-localized kinetochore can be found. According to FEDERLEY (1943, 1945), »bei den Lepidopteren gibt es nichts, das darauf deuten würde, dass jedes Chromosom ein besonderes genau lokalisiertes Zentromer besässe» (1945, p. 9).

In the findings of OKSALA (1943) on Odonata, too, there is evidence of a non-localized kinetochore. Not only in some species have the bivalents at metaphase I their longitudinal axis disposed at right angles to the spindle, but from some bivalents more than one chromosomal fibre could also be seen running towards the same pole.

The case reported by PIZA (1939, 1941, 1949) in the Brazilian scorpion *Tityus bahiensis* shows that the chromosomes have not one localized kinetochore in this species. PIZA claims that the chromosomes of *Tityus* have two terminal kinetochores, because they not only separate parallelly at anaphase, but also during their anaphase movement their ends turn to the poles, becoming somewhat concave. SCHRADER (1947) is inclined to consider that the chromosomes of *Tityus* have a diffuse or multiple kinetochore instead of two terminal kinetochores.

Not only in animals but also in plants, there are indications or observations of a non-localized kinetochore. In the Phycomycetae (*Spongospora*), HORNE (1930) reported that at metaphase of mitosis the chromosomes show a disposition perpendicular to the axis of the spindle

and that at anaphase there is a parallel disjunction of these chromosomes.

GEITLER (1930) observed in somatic divisions of *Spirogyra crassa* a parallel anaphasic disjunction of the chromosomes.

Flowering plants also show this phenomenon. In the family Juncaceae, MALHEIROS, CASTRO and CAMARA (1947) reported the existence of chromosomes without a localized kinetochore in *Luzula purpurea*. In the somatic divisions, at metaphase, the chromosomes disposed themselves at right angles to the axis of the spindle, and separated parallelly, accomplishing the anaphasic movement with their ends slightly turned to the poles. At both divisions of meiosis they performed the anaphasic movement in the same way. This case suggests the existence of a diffuse kinetochore.

TABLE 1. *Organisms in which the existence of a non-localized kinetochore has been suggested* (○), *observed* (+) *or experimentally established* (*).

Organisms	Authors	Symbols
ANIMALS		
Hemiptera		
Tamalia coweni	RIS (1942)	*
Steatococcus tuberculatus	HUGHES-SCHRADER and RIS (1941)	*
Nautococcus schraderae	HUGHES-SCHRADER (1942)	+
Protortonia primitiva	SCHRADER (1931)	+
Llaveia bouvari	HUGHES-SCHRADER (1931)	+
Llaveiella taenechina	HUGHES-SCHRADER (1940)	+
Protenor belfragei	SCHRADER (1935)	+
Gerris	GEITLER (1938)[1]	○
Lepidoptera	FEDERLEY (1943, 1945)	○
Odonata	OKSALA (1943)[1]	○
Acari		
Pediculoides ventricosus	PĂTAU (1936)	○
Pediculopsis graminum	COOPER (1939)	○
Scorpionida		
Tityus bahiensis	PIZA (1939, 1941, 1949)	○
PLANTS		
Spongospora (Phycomycetae)	HORNE (1930)	○
Spirogyra crassa	GEITLER (1930)	○
Luzula purpurea (Juncaceae)	MALHEIROS, CASTRO and CAMARA (1947)	+

[1] The writer has not had an opportunity to consult the papers of GEITLER (1937) and OKSALA (1945).

These observations show the wide occurrence of the non-localized kinetochore, not only in whole groups of animals but also among plants. Its experimental demonstration, effected with the help of X-rays, confirms the idea that we are not dealing with the orthodox type of localized kinetochore.

The study carried out on the organisms possessing a non-localized kinetochore has revealed two features of particular interest for the understanding of the origin and evolution of kinetochores. In these organisms the property of a special cycle of division, present in the localized kinetochore, seems in many cases to be absent or much reduced; secondly, not all chromosomes of an organism, and not all parts of a single chromosome, seem to have the same potentiality of active mobility on the spindle.

Particularly interesting in connection with this first question is the meiotic behaviour of the chromosomes of the male *Llaveiella taenechina* (HUGHES-SCHRADER, 1940). The autosomes show a perfect separation into chromatids at metaphase I and each pair of half-chromatids produces an individual spindle (the division into chromatids being apparently accompanied by the synchronic division of the kinetochore). HUGHES-SCHRADER (1942) has observed in *Nautococcus schraderae* that the valence of the chromosomes during division was also modified; at somatic metaphase a clear four-parted condition of the chromosomes was observed, at telophase a particularly wide separation was visible and no matrical material connecting the half-chromatids was seen. The same valence was observed in meiotic chromosomes. Similar conditions were observed in *Protortonia primitiva* by SCHRADER (1931), in *Luzula purpurea* by MALHEIROS, CASTRO and CAMARA (*l. c.*), in *Steatococcus* by HUGHES-SCHRADER and RIS (*l. c.*), and in *Tamalia* by RIS (*l. c.*). RIS considers this complete separation of the chromosomes into chromatids to be a »result» (p. 275) of the diffuse kinetochore. I think that it is not necessarily a result, since coexistence does not necessarily mean direct connection. There is no obvious reason that can prevent us from thinking that a diffuse kinetochore would not be able to hold two chromatids together as does the localized kinetochore, the only difference would be that it would hold them throughout their whole length instead of at a single region. The complete separation of the chromatids simply shows that the property of a special cycle of division characteristic of the localized kinetochore is absent or much reduced in the chromosomes of these organisms.

HUGHES-SCHRADER (1942) observed that the anaphasic separation

of the chromatids was usually asynchronous in the first division of the spermatocyte of *Nautococcus schraderae*. It was characterized by very marked differential rates in the separation of the chromatids; in contrast, the second anaphase and the somatic divisions showed almost invariable synchrony of disjunction. HUGHES-SCHRADER (1940) has observed the same phenomenon in *Llaveiella taenechina*; at anaphase I the pairs of half-chromatids also proceeded in their poleward migration at individually different rates.

These observations indicate that in the same organism not all chromosomes possess the same potentiality of active mobility. If fragmentation of chromosomes occurs in these species, as is possible owing to the diffuse nature of the kinetochore, new chromosomal races that can arise as a result of this fragmentation may get chromosome complements with different potentialities of active mobility on the spindle.

Not only between the chromosomes of the same organism can there be found a difference in the degree of active mobility, but also, in some species, the different parts of the same chromosome have different active mobility. It is a common feature of some of the cases just reported that during anaphase the chromosomes become somewhat concave, turning their ends towards the poles. This feature has been observed by PIZA (*l. c.*) in *Titygus*, by MALHEIROS, CASTRO and CAMARA (*l. c.*) in *Luzula*, by HUGHES-SCHRADER and RIS (*l. c.*) in *Steatococcus*, and by RIS (*l. c.*) in *Tamalia*. In this last organism RIS has not only observed the curvature of the ends of the chromosomes, but also the curvature of the whole daughter plate. He has explained this bending of the ends of the chromosomes and of the plate by assuming a proportional shortening of the chromosomal fibres during anaphase; all parts of an individual chromosome would then tend to be equally distant from the pole, the same being true of all chromosomes. This is undoubtedly a possible explanation, but the features exhibited by the *X* chromosome of *Protenor* and by the big chromosome of *Thrichiura* cannot be explained in this way; on the contrary, they favour the assumption that inside the same chromosome different regions may possess different potentialities of active mobility. SCHRADER (1935), studying the *X* chromosome of *Protenor*, observed that in the first spermatocyte division this chromosome showed »its daughter halves proceeding toward the poles broadside on, with little or no bending of the body of the chromosome». In the second spermatocyte division the *X* had quite a different behaviour: »in its anaphase movement its long axis is parallel to the axis of the spindle instead of at right angles». In *Thrichiura* FEDERLEY (1945) observed

that at metaphase I the end of the big chromosome was drawn towards the pole in some cells while in others it was the middle of the same chromosome that was thus drawn.

Especially the case of *Protenor* shows that, according to different physiological conditions existing inside the same individual, the different regions of the same chromosome (possessing a non-localized kinetochore) react in different ways. If this differential behaviour is not the result of a position effect, the occurrence of chromosome fragmentation in these species will lead to fragments exhibiting different degrees of active mobility on the spindle, and consequently there can exist here one more possibility for the establishment of chromosomal races with kinetochores possessing different potentialities of active mobility. The cases reported above of species in which the different chromosomes of the same chromosomal set proceed in their anaphasic movement at different rates show that such a differential behaviour of active mobility can become established.

A particular instance requiring mention is the case of *Ascaris megalocephala*. The chromosomes of this organism are polycentric. During the cleavage divisions, the chromosomes in the cells which will form somatic tissues undergo a peculiar fragmentation; each chromosome (two or four according to the variety) breaks up into a number of portions. While the end-regions are left in the cytoplasm and degenerate, as acentric fragments, the median portions behave as entirely separate chromosomes. Before breakage it can be seen that the median part of the chromosome shows chromosomal fibres but that the end-regions are devoid of such connections with the poles. These chromosomal fibres of the median region are not spread over the body of the chromosome in the same way as in the case of the diffuse kinetochore; each detached median portion carries only one kinetochore.

Three main types of kinetochores can thus be recorded among living organisms, *viz.*

(1) The localized kinetochore. Known in most plants and animals.
(2) The diffuse kinetochore. Known among some of the organisms listed in Table 1.
(3) The multiple kinetochore. Known in *Ascaris megalocephala.*

The structure of the centromere of rye chromosomes, and the previously gathered evidence concerning the origin of new kinetochores, are very helpful for the understanding of the nature and evolution of the multiple and diffuse kinetochores.

HUGHES-SCHRADER (1942), as reported before, has observed in *Nautococcus* that the half-spindle fibres arise from a longitudinal zone coextensive with the entire length of the chromosome (this observation was made at late diakinesis). From this observation and from X-rays experiences it has been ascertained that the entire chromosome body is able to produce chromosomal fibres. The fact that it is chromosomal fibres that are seen and not a chromosomal sheet leads me to think that it may not be all the chromomeres of the chromosome that are involved in the process of forming the half-spindle component, but that the property of active mobility may be only localized in some of the chromomeres. These chromomeres may be many and may have a few chromomeres of the normal type between them in such a way that when the chromosome is already rather contracted at late diakinesis (as in the case studied) the chromosomal fibres will appear close together, not leaving so much space between them.

In most cases in which a diffuse kinetochore has been found the property of a special cycle of division was much reduced or did not exist. In these cases it is possible that the series of chromomeres possessing active mobility have originated by a process similar to the one which has given origin to the T-ends found in rye and maize. Both the T-ends and the diffuse kinetochore exhibit in full the property of active mobility, but the property of a special cycle of division cannot be detected in them. Both are also affected by the physiological conditions present in the cell (at least in some cases, *e. g.*, *Nautococcus*). In the T-phenomenon the property of active mobility is only exhibited at meiosis.

We may assume — as the property of a special cycle of division does not seem to be always necessary for the normal performance of the chromosome movements during division — that in the process of chromosome evolution the property of active mobility has established itself firstly in a chromomere and subsequently in several chromomeres of the same chromosome, only later being accompanied by the property of a special cycle of division. According to this assumption, a diffuse kinetochore has arisen when most of the chromomeres of a chromosome have acquired the property of active mobility, and a localized kinetochore when a restricted group of chromomeres have acquired separately the properties both of active mobility and a special cycle of division. In species where both properties have had the possibility of establishing themselves at more than one separated region of the chromosome, a multiple kinetochore like that found in *Ascaris megalocephala* has originated.

If it seems probable that in the evolution of chromosomes the property of active mobility has established itself first and that, in certain cases, the property of a special cycle of division has afterwards become added to the division mechanism, this does not imply that owing to subsequent transformations the reverse process could not have taken place. Mitosis and meiosis are mechanisms controlled by many factors having definite relations to each other that result in a balanced system, although »deviations» from the general scheme are frequently able to establish themselves successfully.

Lastly, it is now well established that the specialized region responsible for the movement of chromosomes during division does not display the same features in all organisms. In most organisms studied the property of active mobility on the spindle is confined to a single region, in many others it is diffuse throughout the whole chromosome, and in others, again, it has a multiple distribution.

These types do not seem to represent distinct formations that are sharply separated or have no phylogenetic connection with each other, but they can be considered as differentiated forms of the same process. These different types are not themselves constant, and exhibit dissimilar features in different organisms.

IV. NAVASHIN'S THEORY.

In 1932 NAVASHIN refuted the idea that new chromosome numbers could arise by simple fragmentation or fusion of chromosomes, independently of the number of kinetochores present in the chromosome set. He clearly developed the idea that only chromosomes possessing one single kinetochore can perpetuate themselves through a considerable number of cell generations and that acentric and polycentric chromosomes cannot do so. Secondly, he stated that kinetochores do not arise *de novo* and do not lose their properties in the course of evolution. He enunciated these two statements in the following way: »the chromosome number is conditioned by the number of functional kinetic bodies, the latter not being subject to *de novo* formation» (p. 226). The theory of NAVASHIN has been of great value, inasmuch as it has called the attention of the cytologists to the important rôle played by the localized kinetochore in the chromosome movements.

The validity of the first statement of NAVASHIN as regards the chromosomes possessing a localized kinetochore was confirmed immediately

after the publication of his paper by MATHER and STONE (1933) as well as by subsequent works.

The second statement, *viz.* that kinetochores do not arise *de novo*, has been generally accepted owing to the fact that evidence to the contrary was not found in the years closely following NAVASHIN's work.

Several facts have also contributed to strengthening this idea. The results of crossing-over studies have contributed indirectly to supporting the idea that the centromere was a genetically inert body, fundamentally distinct from the arms of the chromosome, owing to the fact that crossing-over per unit cytological length near the centromere is lower than elsewhere. The morphological aspect of the centromere has afforded a main contribution to the general acceptance of this idea. The structure of the centromere has proved to be difficult to analyse, the centromere being described mainly as a »gap».

These facts have established in the mind of most cytologists the concept that the localized kinetochore is a specialized »body» constant in its properties, not subject to hereditary changes or evolution and having a distinct nature when compared with the chromosome arms.

The theory of NAVASHIN has proved to be very fruitful, even though its universality can no longer be accepted. The evidence accumulated during the forties has definitely shown that it could not be maintained *in toto*. Moreover, if with NAVASHIN it is considered that kinetochores do not arise *de novo*, it is necessary to assume that all existing kinetochores derive from a single kinetochore that has been formed once, the mechanism by which this first kinetochore has been formed not having been repeated any more. That the localized kinetochore has up to now been regarded as a rather stabile region of the chromosome body, cannot be denied, but nor do we know to what extent this idea depends on the methods used at present.

The general applicability of the statement that new chromosomes cannot arise by simple fragmentation of other chromosomes independently of the number of kinetochores present in the chromosome set can no longer be maintained after the experiences of HUGHES-SCHRADER and RIS (*l. c.*) reported above. Several workers, among them FEDERLEY (1945), have observed indications that fragmentation of chromosomes occurs in natural conditions among organisms where a non-localized kinetochore is supposed to exist.

All the evidence enumerated in the previous pages shows that the second statement of NAVASHIN, *viz.* that of the constancy, and absence of *de novo* formation of kinetochores, is put in doubt by numerous facts.

CAROTHERS (1936) was led to assume that the centromere was not a specialized region of the chromosome but only a temporarily modified chromomere. »In my opinion», says CAROTHERS, »a specialized chromomere of each chromatid normally sends out a fine pseudopodial-like process at the metaphase which becomes the chromosomal fiber, but a change of rate of condensation and diffusion may result in a shifting of this function to a more precocious chromomere» (p. 489). In the opinion of CAROTHERS it is a simple variation in the rate of »condensation and diffusion» in the chromosome that is responsible for the creation of new kinetochores. This idea is not supported by any known evidence.

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SUMMARY.

(1) The localized kinetochore of the chromosomes of *Secale cereale* L. may be called a *reversed repeat* owing to (a) its microscopic structure, (b) the normal behaviour during division of iso-fragments arising by misdivision of the centromere and (c) the close association of the two halves of the centromere one with the other in iso-fragments showing inter-arm pairing.

(2) There is evidence suggesting the existence of genes at the centromere.

(3) The localized kinetochore possesses two properties: active mobility on the spindle and a special cycle of division. Regions of chromosomes independently possessing these two properties exist in rye and other organisms; and there is no evidence supporting the idea that the regions in question receive their origin from the centromeres already existing. There is a possibility that they could arise at the same spot in the chromosome, giving origin to a new localized kinetochore.

(4) In some species having chromosomes with a diffuse kinetochore, not all the chromosomes of the same individual possess the same degree of active mobility. If fragmentation of chromosomes occurs in these species, as is possible owing to the diffuse nature of the kinetochore, new chromosomal races that can arise as a result of this fragmentation may get chromosome complements with different potentialities of active mobility on the spindle.

(5) Both the »T-ends» and the diffuse kinetochore exhibit in full the property of active mobility (the T-ends only at meiosis), but the property of a special cycle of division cannot be detected in either of them.

(6) The property of active mobility seems to have established itself, firstly, during the process of chromosome evolution, in one or several chromomeres, a diffuse kinetochore having arisen when most of the chromomeres of a chromosome have acquired this property. The subsequent establishment of the property of a special cycle of division has given rise to the formation of chromosomes with localized or multiple kinetochores.

(7) These types of kinetochores do not seem to represent distinct formations that are sharply separated or have no phylogenetic connection with each other, but they can be considered as differentiated forms derived from the same process.

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LUZULA AND THE MECHANISM OF CHROMOSOME MOVEMENTS

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I. INTRODUCTION.

FIBRILLAR structure is one of the fundamental properties of the spindle of nuclear division. The opinion sometimes put forward that the fibrous structure is not »real» but only »a fixation artefact» cannot be taken seriously. It is true enough that the spindle fibres often are to be seen with a much coarsened appearance after bad fixation and that after good fixation there is sometimes very little to be seen of the fibres. But it must equally be realized that the fibrous structure as it is brought out in fixed preparations cannot have arisen out of nothing. We can be fairly safe in interpreting it as the result of some kind of submicroscopic fibrillar structure existing already in the living cell and coarsened by precipitation at fixation. Evidence for the existence of a fibrous structure of the spindle in the living cell is given especially by studies with polarized light (*e. g.*, SCHMIDT, 1939; HUGHES and SWANN, 1948), which show double refraction in the living spindle. The evidence for »the reality of the spindle fibres» is summarized by SCHRADER (1944).

The existence of a fibrous structure in the spindle is one of the facts upon which the traction fibre hypothesis of the chromosome movements is founded. According to this hypothesis, the centromeres of the chromosomes are connected to the spindle poles by contractile fibrils which by their contraction pull the chromosomes to the poles during anaphase. A summary of the evidence in favour of the traction fibre hypothesis is given by CORNMAN (1944). His arguments give strong support to this hypothesis and it is obvious that certain fundamental points of the traction fibre idea must *in one way or another* form a part of any fertile hypothesis about the movements of the chromosomes.

The behaviour of the chromosomes on the spindle demonstrates that the centromeres are attracted to the spindle poles by forces increasing in strength with an increasing distance between centromere and pole and that each centromere is attracted only towards that pole towards which it is turned; the anaphase centromere at mitosis is turned towards

one pole only and the mitotic metaphase centromere consists essentially of two anaphase centromeres turned towards opposite poles. These forces explain the movements of the chromosomes to the equator at prometaphase and those to the poles at anaphase equally well. The chromosomes behave very much as if they were connected with the poles by attenuated rubber bands. Evidence: The equilibrium of trivalents at metaphase (ÖSTERGREN, 1945 a; BÖÖK, 1945), the transverse equilibrium of the chromosomes on the spindle (ÖSTERGREN, 1945 b), and the behaviour of T-chromosomes in rye (ÖSTERGREN and PRAKKEN, 1946); *cf.* also the summaries by ÖSTERGREN (1949) and PRAKKEN (1948). These results also point towards *something like* a traction fibre for their explanation.

In this connection attention must also be drawn to a characteristic feature in the arrangement of the fibrillation of the spindle, *viz.* that some of the fibrillar structure is attached to the centromere, forming what looks like a bundle proceeding from the centromere towards the pole; »the chromosomal fibre» or »half spindle component», as it has been called by SCHRADER (*e. g.*, 1944). These chromosomal spindle fibres or fibre bundles grow gradually shorter during anaphase as the daughter chromosomes approach the poles. It is very easy to suggest, as often has been done, that this structure is an important part of the machinery moving the chromosomes, and that these fibres actually pull the chromosomes by their contraction. Some support for this opinion is given by the fact that similar spindle fibres are attached also to the T-ends in rye, which chromosome ends behave as centromeres at meiosis (ÖSTERGREN and PRAKKEN, 1946; concerning the T-ends see also PRAKKEN and MÜNTZING, 1942). In organisms which show kinetic properties all along the length of their chromosomes (»diffuse kinetochore» or »diffuse centromere») there are also found spindle fibres attached all along the length of the chromosomes, a situation which is best known from the Homoptera and Heteroptera (*e. g.*, HUGHES-SCHRADER, 1948; HUGHES-SCHRADER and RIS, 1941; RIS, 1942).

There are thus some facts that indicate that the chromosomes may be moved simply by the contraction of contractile protein fibrils, which *e. g.* may contract by folding up their polypeptide chains, as suggested by SCHMIDT (1939). But it must also be realized that there are other facts that clearly demonstrate that the mechanism is hardly quite as simple as that. Evidence of special importance against the simple traction fibre idea is given by the observations that bodies can move

fairly freely in a transverse direction in the spindle straight across the presumed traction fibres.

II. THE MOBILITY OF BODIES ACROSS THE SPINDLE FIBRES.

First a brief reference may be made to the works on microdissection of the spindle by CHAMBERS (1924) and WADA (1935). Both authors investigated the spindle by moving needles in it. Their results were somewhat variable and do not contribute much to answer the question of whether or not the spindle contains continuous fibrils connecting the chromosomes with the poles. It is obvious that the method is a very crude one, and one may be well justified in having serious doubts concerning its applicability to the present question, although it may still contribute valuable information on some other properties of the spindle.

More promising are those microdissection experiments in which the chromosomes themselves perform the dissection of the spindle during the course of a normal nuclear division. There are data indicating that the chromosomes have a fairly high degree of freedom in moving in a transverse manner across the fibrillar structure of the spindle. The existence of transverse equilibria on the spindle, such as described by ÖSTERGREN (1945 b), *e. g.* leading to an assortment of the chromosomes according to their size, the big chromosomes on the periphery and the small ones in the centre of the plate, is in itself a good indication that the chromosomes can move in a transverse direction in the spindle.

A study of the prometaphase events in higher plants (*e. g.*, SCHAEDE, 1938, and unpublished observations by the present writer) shows that at a certain stage of mitosis the chromosomes assemble in an irregular ball in the centre of the spindle, and later (at metaphase) they can be found more evenly distributed over the plate, a change no doubt involving transverse movements through the spindle. These movements are especially obvious in the case of univalents at meiosis, which in prometaphase are found in the centre of the spindle together with the paired chromosomes, while the univalents at metaphase, on the other hand, usually are »floating» on the surface of the spindle (personal observations on various materials in the Triticinæ). Such cases show fairly convincingly that the chromosomes can move transversely through the »general ground substance of the spindle», but they scarcely prove that objects can pass through the chromosomal spindle fibres attached to the centromeres and presumably acting as traction fibres, as it may be

suggested that the chromosomes somehow succeed in performing their movements between these fibres. A careful study of the prometaphase events, especially in films of living mitosis, might make it possible to remove this opposition. Such studies may not be very easy to perform, if possible at all, so it will in any case be well justified to look also for indirect evidence of a more conclusive kind than that just mentioned. Such evidence is given by the chromosome behaviour in *Luzula purpurea*.

III. THE LUZULA CASE.

One of the most sensational events in cytology in recent years was the discovery (MALHEIROS and CASTRO, 1947; MALHEIROS, CASTRO and CAMARA, 1947; CASTRO, CAMARA and MALHEIROS, 1949) of the existence of a diffuse centromere in a plant genus, *viz.* in *Luzula*. The species in which this was first found and where it is most easily studied is *L. purpurea* LINK. This species differs from most species of the genus by the fairly big size of its chromosomes (length at somatic mitosis ca. 6 μ; MALHEIROS, CASTRO and CAMARA, *l. c.*) and by the fact that its chromosome number is as low as $n = 3$.

The chromosomes of this species show centromere activity all along their length, as demonstrated by the following data (*cf.* the figures and descriptions of MALHEIROS, CASTRO and CAMARA, 1947; CASTRO, CAMARA and MALHEIROS, 1949).

(1) The chromosomes do not show any morphologically visible centromere constriction.

(2) The whole chromosome body is lying flat in the equatorial plane during metaphase. This is scarcely caused merely by stiffness as might be suggested, because the chromosomes are usually bent in other directions.

(3) Chromosomes having a localized centromere regularly manifest the characteristic effects of the forces giving the »transverse equilibrium» (ÖSTERGREN, 1945 b), the chromosome region containing the centromere striving centripetally on the metaphase plate and the rest of the chromosome body striving centrifugally. The study of metaphase plates of *Luzula purpurea* in polar view shows, however, no indications of such an effect. The chromosome ends show here no tendency to be expelled out of the spindle into the cytoplasm as in the case of ordinary chromosomes.

(4) At the anaphase separation the daughter chromosomes move

apart at first parallel to one another, and in the later stages both ends of each chromosome lead in the poleward migration.

(5) Of decisive importance is the fact that the fragments obtained by x-ray breakage of the chromosomes show normal anaphase movements (CASTRO, CAMARA and MALHEIROS, 1949).

Besides these characteristics the cytology of the *Luzula* material also shows some other peculiarities, which will not be considered here as they seem to be of minor concern for the main purpose of the present paper. Here belongs especially the strange chromosome behaviour at meiosis.

I have had an opportunity myself to study the cytology of *Luzula purpurea*. My material was derived from seeds obtained from the Botanical Garden of Coimbra, Portugal. Root tips were fixed in chrom-acetic-formalin (Navashin, as modified by MÜNTZING, 1933), in Benda, in Helly, and in the glycerol mixture of ÖSTERGREN (1947), which was suitable for bringing out the centromere structure in *Hyacinthus* and *Allium*. The three first mentioned fixatives gave satisfactory results, Benda seemed to be slightly better than the others. The glycerol mixture did not, unfortunately, reveal anything concerning the organizational details of the diffuse centromere, and the fixative is not very good for general cytological purposes, but the slides could still be used to confirm the results obtained after the other fixations. Flower buds were fixed in chrom-acetic-formalin after prefixation in acetic alcohol. All material was embedded in paraffin and sectioned. Some slides were stained by crystal violet for chromosome study and others by acetocarmin or by a 45 % acetic acid solution of alizarin viridin (0,25 %) for studies of the spindle; in the latter case very thin sections must be used because of the high intensity of the stain.

The characteristics of the *Luzula* mitosis mentioned above are nicely confirmed by my material. My studies on this species have been centered on the behaviour of the chromosomes at mitosis and the events of meiosis will not be considered in the present paper. Both root tip mitosis and the first mitosis in the pollen grain have been studied.

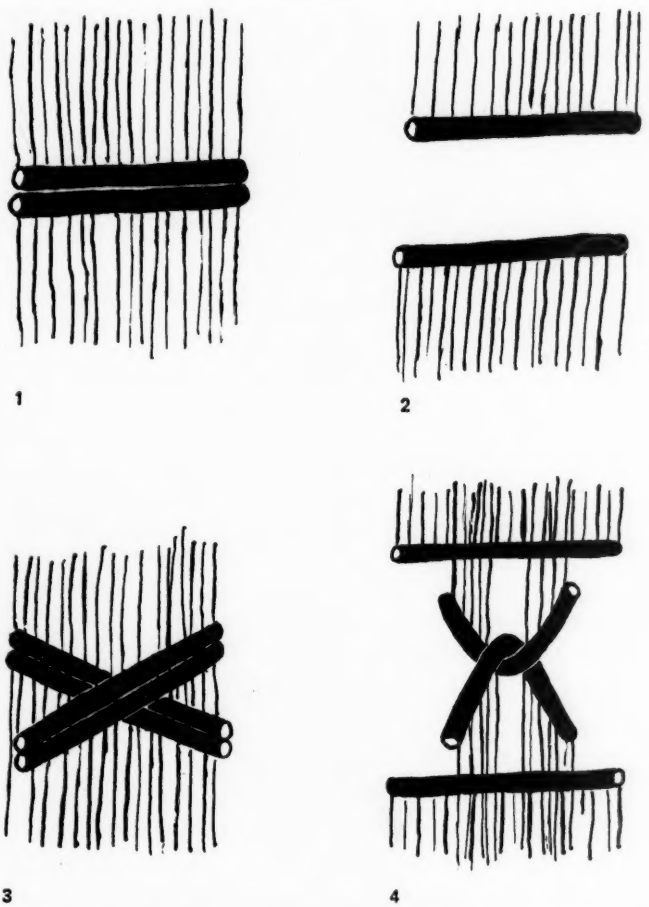
The arrangement of the chromosomes in the metaphase plate is demonstrated by Figs. 5—16, and the parallel separation of the daughter chromosomes by Figs. 17—18. According to the Portuguese authors, the chromosome ends are preceding the rest of the chromosomes in the poleward movement during late anaphase. This effect is quite weak at the mitosis studied in my material, but the existence of tendencies of this kind can be noticed. The description »parallel separation» accounts

best for the anaphase in the present case. There is one respect in which my material differs somewhat from that of CAMARA and his co-workers. They found that during the metaphase of the first pollen mitosis the chromosomes were curved with their ends in close proximity to one another, each chromosome thus forming a ring. This is not the case at the pollen mitosis of my material. Here the chromosomes are straight or irregularly bent in various directions, just as they are at the somatic mitosis. The difference may well be caused by some genotypic differences or by different conditions of cultivation (or both).

There is a special feature sometimes found in the arrangement of the chromosomes in the metaphase plate, *viz.* they can lie in a cross one over another (Figs. 7—8, 10, 13 and 16). This arrangement gives important information on the constitution of the spindle fibres; and it was actually the observation of this arrangement that led to the writing of the present paper. Such a crosswise arrangement of the chromosomes can also be seen in three of the nine somatic metaphase plates depicted by MALHEIROS, CASTRO and CAMARA (1947, Text-figs. 1—3 and Figs. 1—4 on Plate I). These authors do not make any comments on this fact.

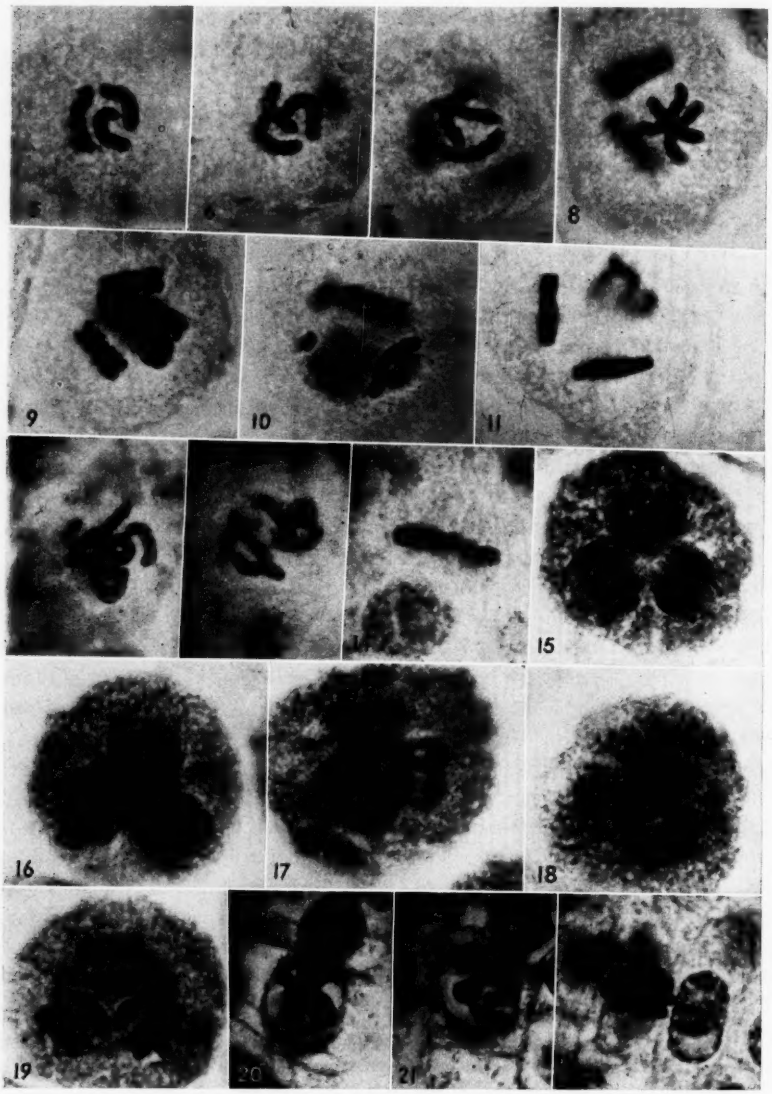
Such an arrangement implies an interlocking of chromosomes and spindle fibres in a manner that would cause serious trouble to a regular anaphase separation (Figs. 1—4). If it is found that no such anaphase troubles occur, the obvious conclusion will be that the chromosomes are capable of moving transversely through those spindle components that act as traction fibres of the chromosomes. To make that conclusion it is, of course, necessary to demonstrate that the chromosomes are attached to the spindle at the stage when these cross arrangements are found, and furthermore a sufficient number of anaphases must be studied to exclude the possibility that the disturbances were missed by a mere accident; the number of anaphases that must be studied to this end will depend on how often the cross arrangement occurs at metaphase.

In my material of root tip mitoses there were 12 metaphases showing a cross arrangement of two or more chromosomes out of a total number of 43 metaphase plates (*i. e.* a frequency of about 25—30 %). All the anaphases in this material (= 109) were normal. The metaphases were studied in polar view and the anaphases in side view. The metaphase distribution of 12 : 31 was compared to the anaphase one of 0 : 109 by the method for »the exact treatment of 2×2 tables» (FISHER, 1938, paragraph 21:02). The probability for an identity of the two distributions was found to be $p = 7,5 \times 10^{-8}$. The ordinary χ^2 test was not



Figs. 1—4. Schematical representation of metaphase and anaphase to explain the events that might be expected from a crosswise arrangement of the chromosomes in an organism with a diffuse centromere. The subdivision of the chromatids into half-chromatids has not been shown in the figure and only the chromosomal spindle fibres have been drawn. — Figs. 1—2, mitosis as it normally takes place. — Fig. 3, a crosswise arrangement of two chromosomes at metaphase, an arrangement often found in *Luzula*. — Fig. 4, the anaphase arrangement expected to follow after the stage of Fig. 3; this arrangement was never found.

used in the present case as the number of expected cases in one of the groups was less than five. To this can be added the evidence that during



Figs. 5—22. Photographs of various mitotic stages in *Luzula purpurea*. — Figs. 5—11 and 15—19, first pollen mitosis. Note that this division takes place in pollen grains united in tetrads, consequently many figures show three different mitoses at the same focal level (*cf.* MALHEIROS, CASTRO and CAMARA, 1947). — Figs. 12—14, root tip

prometaphase, when the spindle fibres have already attached to the chromosomes, there is found an even much higher frequency of crosswise arrangement of the chromosomes (*cf.* below).

A study of somatic mitoses in various parts of the floral buds gave the following numbers: No metaphases with a crosswise arrangement were found among 20, and all the 17 anaphases studied were normal. This represents a significantly lower frequency of the crosswise arrangement than in the root tips; a value of $p = 0{,}0058$ was found by the same method as used above when the root tip metaphases were compared to these metaphases. Still, there must also in these mitoses be a considerable movement of chromosomes across the chromosomal spindle fibres of their fellows as revealed by studies of the prometaphase, which here takes the same course as in the root tips. But these movements would then seem to be finished earlier in the floral tissues, so that a clean arrangement is reached at metaphase.

The observations on the first mitosis in the pollen grains gave the following numbers: 8 metaphases with a crosswise arrangement were found in a total of 82 plates studied, and all the 95 anaphases observed were normal. The distribution was tested as above for the root tips, and the value $p = 0{,}0018$ was obtained.

The metaphase plates treated above were considered to belong to the metaphase stage because the chromosomes were arranged in the equatorial plane. Obviously the flat arrangement in the equatorial plane cannot be quite as perfect when chromosomes cross one another as when they do not, and in a few cases some slight deviations from the equator-

mitosis. — Figs. 20—22, mitosis from somatic tissue of the floral buds. — Figs. 5—8, metaphase in polar view. — Figs. 5—6, the ordinary arrangement of the chromosomes. — Fig. 7, one chromosome is lying across another. — Fig. 8, one chromosome is lying across two others. — Fig. 9, a very early stage of anaphase, the chromosomes seen in optical cross section. The subdivision in half-chromatids is indicated. — Fig. 10, a metaphase in side view showing an arrangement similar to that seen in polar view in Fig. 8. — Fig. 11, two ordinary metaphases in side view. — Fig. 12, a metaphase in polar view with the ordinary arrangement of the chromosomes. — Fig. 13, a metaphase with one chromosome lying across another. — Fig. 14, a metaphase in side view. — Fig. 15, two metaphases in side view showing the spindle fibres. — Fig. 16, in the upper metaphase one chromosome is lying across another; note that the spindle fibres are attached to the chromosomes. — Fig. 17, early anaphase, showing empty regions between the separating daughter chromosomes. — Fig. 18, late anaphase. — Fig. 19, telophase with phragmoplast. — Figs. 20—21, prophases with polar caps of spindle substance. — Fig. 22, prometaphase showing helter-skelter arrangement of the chromosomes inside the spindle. — All figures are from material fixed in chrom-acetic-formalin; in the case of the flower buds in combination with a prefixation in acetic alhocol. — Figs. 5—14, stain: crystal violet. — Figs. 15—22, stain: acetocarmin. — × 1330.

ial arrangement were noticed also in other chromosomes in these plates. The question of whether the plates may be called typical metaphases or not is a purely nomenclatorial one without any bearing on the essential points of the present paper. Of much more importance is the question of whether or not the chromosomes are attached to the spindle at this stage.

A study of the development and organization of the spindle in the root tip mitoses gave the following results: The first indications of the spindle are found at late prophase, when there are organized polar caps (»Polkappen») of spindle substance on two opposite sides of the nucleus. Later on the nuclear membrane is dissolved and the spindle invades the nuclear region. The chromosomes are clustered in an irregular ball in the centre of the spindle, probably pushed into this position by the invasion of the spindle substance. After this stage the chromosomes appear to be more spread out over the spindle, but still lying in an irregular way; at this stage the spindle fibres start to be attached to the chromosomes. This stage is gradually transformed into full metaphase by the movement of the chromosomes to the equatorial plane. The prometaphase events are thus in good agreement with the ordinary type characterizing higher plants, such as it is described *e. g.* by SCHAEDE (1938), naturally with the difference that the chromosomes here show centromere activity all along their length.

At metaphase the chromosomes show spindle fibres attached all along their length. This is the case also in those metaphases where there is a crosswise arrangement of a pair of chromosomes. As a matter of fact, the chromosomes are attached to the spindle already in much earlier stages, during prometaphase when they lie distributed more or less at random (»helter-skelter») in the spindle. In these early stages there is much more cross arrangement of the chromosomes than at metaphase; no exact counts of the frequency were made, but this arrangement would seem to exist in practically every cell at this stage. Thus already during the prometaphase movements there must be a considerable transverse movement of the chromosomes through the chromosomal spindle fibres of their fellows before the metaphase position is reached, and these movements continue during metaphase until finally the chromosomes reach a position allowing them a clean anaphase separation.

The development of the spindle and the course of mitosis are quite the same in the somatic mitosis of the floral buds as was just described for the root tips. Figs. 20—22 are from such mitoses.

The development of the spindle at the pollen mitoses, on the other hand, seems to deviate slightly. In the pollen grains I have not been able to detect any polar caps of spindle substance outside the prophase nuclei. However, in these mitoses, too, there is a helter-skelter arrangement of attached chromosomes during prometaphase, and it can be clearly seen that the chromosomes found in a crosswise arrangement at metaphase are furnished with chromosomal spindle fibres (Fig. 16).

A few words may also be mentioned on some other features of the spindle figure. At metaphase the spindle is barrel shaped with only a very weak convergence towards the poles (as already reported by MALHEIROS, CASTRO and CAMARA, 1947). The spindle is more bi-acuminate during prometaphase (Fig. 22), and during early anaphase the convergence is also stronger than at metaphase. I have seen similar changes in spindle shape in other plants, so they are not specific for *Luzula*. Concerning their cause, it is scarcely possible to make any suggestions at the present state of our knowledge. The spindle region between the chromosome groups at anaphase also shows a fibrillated structure and this region is later transformed in the usual way into a strongly fibrillated phragmoplast which forms the new cell wall. In the first pollen mitosis, however, the phragmoplast (Fig. 19) does not form any cell wall between the vegetative and the generative nuclei. »Interzonal connections» in the sense of SCHRADER (1944, pp. 34—35), formed between the separating daughter chromosomes by their sticky surface layers, which are so characteristic of the Homopteran mitoses, were noticed in some mitoses of the present case, too. They are especially common in the somatic mitoses observed in the floral parts; in these mitoses the chromosomes are very sticky also at metaphase. Anaphase chromosomes without such »interzonal connections» move just as well as those having this structure, so it is obvious that they are not involved in the mechanism of the chromosome movements. At metaphase it can be seen in some mitoses that there exists a fibrillated spindle substance also at the sides of the chromosomes, passing across the equator without attaching to the chromosomes. This is the kind of structure that sometimes has been called »continuous fibres». One may be well justified in doubting that these fibres are continuous connections reaching from pole to pole of the spindle as may be implied by the term, so I would suggest that they be called with a more neutral name, as *e. g* the »general spindle fibres» or the »general spindle substance». General spindle fibres are probably of universal occurrence, and the fact that they are not to be found in some organisms, such as *e. g.* the Homoptera

(HUGHES-SCHRADER, 1948), is probably due merely to technical difficulties of their demonstration. That seems at least to be the most probable interpretation. Fig. 17 shows these so-called »continuous fibres» at anaphase of the pollen mitosis of *Luzula* passing across the equatorial region between the separating daughter chromosomes. The space through which the sister chromosomes have passed during their separation seems to be empty.

A notable feature in the organization of the *Luzula* chromosomes at mitosis is that the half-chromatids are clearly visible; the metaphase chromosomes thus having a quadripartite structure and the anaphase chromosomes a bipartite one as in certain insects with a diffuse centromere (*e. g.*, HUGHES-SCHRADER and RIS, 1941; RIS, 1942). The half-chromatids in *Luzula* are best seen at early anaphase (Fig. 9); in most metaphase chromosomes their presence is manifested only by the quadrangular shape that the metaphase chromosome is seen to have in optical cross sections. It is not possible from my observations to decide whether the chromosomal spindle fibres attach at the ridges formed by the half-chromatids or in the furrow between them. Nor is it possible to decide if the fibres attach in a quite continuous distribution all along the chromosome length or if they form a series of bundles each with its localized point of attachment.

IV. DISCUSSION.

1. THE CAUSE OF THE TRANSVERSE MOVEMENTS.

From the preceding description it is clear that there exist movements of the chromosomes in a transverse manner across the spindle fibres in *Luzula*, which movements separate chromosomes from one another when they happen to lie crosswise one upon another. These movements must obviously be caused by forces acting in directions perpendicular to the spindle axis, and the question arises concerning the nature of these forces.

To explain these movements it is not necessary to introduce the assumption of any new kind of force not known already from other results. The same forces which bring about the prometaphase movements to the plate and the anaphase movements to the poles should be capable of explaining also the present movements without the introduction of any new assumptions.

When two chromosomes are lying across one another, they will

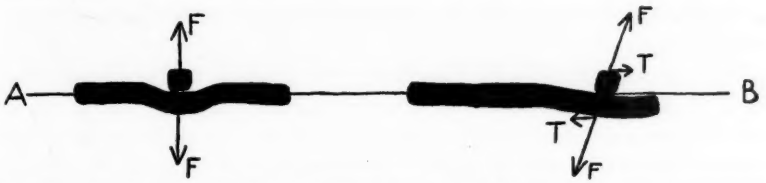


Fig. 23. Schematical drawings to explain the causes of the transverse movements that are assumed to separate chromosomes having a crosswise arrangement at metaphase (or prometaphase). The line A—B represents the level of the metaphase plate. In the left figure the two chromosomes cross one another at their exact centres and the forces (F) by which they exert an elastic pressure upon one another have no transverse components. In the right figure the lower chromosome is crossed off centre, so it will be inclined at the point of contact and F will have a transverse component T. — Note that only the elastic forces by which the chromosomes repel one another, when they are pressed together, have been indicated in the figures. The forces striving to arrange them in the equator, forces which press them against one another, have not been drawn.

come to exert a mutual pressure when they strive to occupy the flat arrangement in the equatorial plane. When the chromosomes cross one another in their exact middle region, the force by which they press upon one another will be perpendicular to the equatorial plane and there should not be any tendency to transverse movements (Fig. 23, the left figure). But when the point of contact does not coincide with the middle in one or both of the chromosomes, their mutual pressure will be more or less skew relative to the equatorial plane (Fig. 23, the right figure). The forces will then contain transverse components striving to separate the chromosomes as indicated in the figure. This follows from the tendency of the chromosomes to arrange themselves in the equatorial plane along their whole length and from their rigidity and elasticity.

It is clear that only rarely will the point of contact between the chromosomes coincide exactly with the middle point of both of them. And, furthermore, even when it does so, this does not represent a stable position; the slightest shift in one direction or another should start the separation mechanism.

2. THE ORGANIZATION OF THE DIFFUSE CENTROMERE.

Another problem that may be considered in the present connection is that concerning the organization of the diffuse centromere. In chromosomes with a localized centromere of the ordinary type, the spindle fibres attach only on the centromere. In the case of chromosomes with the diffuse type of centromere, on the other hand, the fibres attach all

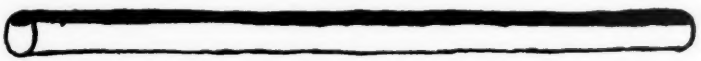
along the chromosome length on those surfaces which are turned towards the poles.

In chromosomes with a diffuse centromere it would appear that each of the two chromatids of a metaphase chromosome has a special side showing kinetic properties, which we may call its front side. The two chromatids of the metaphase chromosome are attached to one another by their back sides, and thus turn their kinetic sides in opposite directions. The anaphase chromosome has spindle fibres attached only to its front side and is pulled by them to the pole towards which this side is turned. In cases where the half-chromatids are spiralized separately (forming two spirals lying side by side), as seems to be the case in *Luzula*, we may then ascribe a similar organization to the half-chromatids.

The question then arises, how it can be possible for a spiralized chromatid (or a half-chromatid) to have a special property on one side, and also the ability to contract to a variable degree by spiralization, without changing this one-sided localization of the kinetic properties. Such an organization could be easily explained by the following hypothesis:

It is assumed that the unspiralized chromonema is itself organized with kinetic properties on one side only, *e. g.* in the form of a kinetic stripe passing along the chromonema (Figs. 24—25). When this chromonema is spiralized, it is coiled in such a manner that it always turns the kinetic stripe in the same direction (Fig. 26). In this manner we obviously get a one-sided localization of the kinetic properties also in the spiralized state of the chromosome. There is good evidence from other data on chromosome behaviour that the chromonemata really are coiled on the basis of such a principle when they spiralize, *viz.* from the fact that two chromatids which have formed a joint spiral still are able easily to separate from one another. This implies that the mutual position of the two chromatids during the coiling is such as assumed above concerning the kinetic side and the rest of the chromonema. Concerning the separation of chromatids that have spiralized together, see *e. g.* GEITLER (1938, p. 65).

Naturally there are many different modifications possible of the basic idea outlined above. Thus we can assume that, instead of a kinetic stripe, the chromonema has a series of chromomeres which have kinetic properties on one side (Fig. 27), or that chromomeres of this type occur intermixed with ordinary chromomeres, or that a chromonema with a kinetic stripe is furnished with a series of ordinary (akinetic) chromo-



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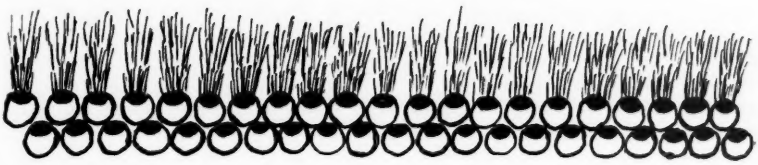

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Figs. 24—27. Schemes explaining the hypothesis on the organization of the diffuse centromere. — Fig. 24, part of the chromonema showing that it has a kinetic stripe (drawn black) running along its whole length on one side. — Fig. 25, a cross section of this chromonema. — Fig. 26, a longitudinal section through a spiralized metaphase chromatid, showing that the chromonema has its kinetic stripe turned towards the same side of the spiral all along the chromosome length; chromosomal spindle fibres attach to the kinetic region. — Fig. 27, an alternative organization of the chromonema, in which it has a series of chromomeres, each with a one-sided localization of the kinetic properties.

meres, *etc.* Suggestions of this kind do not differ in principle from the above hypothesis.

On the basis of the present idea it would not be surprising to find the chromosomal spindle fibres at metaphase localized in a series of bundles, each corresponding to an individual spiral gyre, instead of being distributed in a continuous manner along the chromosome, but,

depending on the details of the chromosome organization, such as the breadth of the kinetic stripe of the chromonema, the situation might also be such as to give the impression of a continuous distribution.

It was suggested by MALHEIROS, CASTRO and CAMARA (1947) that there may be an intimate connection between the peculiar sticky matrix to be found in the chromosomes of *Luzula* and the Hemipteran insects and the diffuse centromere which is also characteristic of these organisms. This idea was worked out in more detail by CASTRO, CAMARA and MALHEIROS (1949) who suggested that the connection consisted in a special modification of the nucleic acid metabolism of the chromosomes, leading to a lower degree of polymerization of the nucleic acids. This was assumed on one hand to make the matrix more sticky and on the other to give it an increased permeability so that the spindle proteins organized by the chromosome could leak out all over the chromosome surface instead of only in a localized region (the centromere) as in ordinary chromosomes. To this idea it must be objected that the essential property of the centromere seems to be that part of the spindle substance is attached to it, and that there does not appear to be any special reason why this property should be connected with a leakage out of the spindle substance at this region. If the spindle substance flows out here there is no reason why it should not afterwards attach itself also at many other places of the chromosomes. Also, as can often be seen when studying the origin of the spindle, the spindle substance does not originate in a morphological connection with the chromosomes, but these are attached to the already formed spindle only at a later stage.

In any event, it is quite probable that the Portuguese authors are right in their idea that there is a close connection between the diffuse centromere and the peculiar development of the matrix in these organisms. I think, however, that the connection between these properties may be due to the following circumstances: In chromosomes of the ordinary type with a localized centromere the chromatids of a metaphase chromosome are firmly associated only in the centromere region, which is carrying the load of the pull working towards the two opposite poles, and in such chromosomes there are no forces striving to separate the chromatids all along their length. In chromosomes of the diffuse centromere type, on the other hand, the poleward pull is working all along the chromosome length, so in this case there is a definite need for a special mechanism keeping the chromatids together in order to prevent a precocious separation. It seems, then, reasonable to suggest that the chromosome matrix in these organisms is specialized for this special function, and that its stickiness is somehow a consequence of this special development.

3. SPINDLE STRUCTURE AND CHROMOSOME MOVEMENTS.

Finally, it remains to discuss the significance that the present results may have for our ideas on the spindle structure and for the explanation of the chromosome movements at mitosis. Any hypothesis on these problems must pay due regard to the fact that the chromosomes are capable of moving in a transverse manner across the fibre structure of the spindle and even through the chromosomal spindle fibres.

Perhaps it might be objected that these chromosomal spindle fibres through which the chromosomes obviously can move are not the real »traction fibres» of the chromosomes, but that these latter are formed only at a later stage when a clean metaphase arrangement has already been reached, *e. g.* just before the start of anaphase. This objection is disposed of by the following reasoning:

Such an opinion would obviously imply that the prometaphase movement to the plate and the anaphase movement to the poles are due to quite different mechanisms. Now, the movement to the equatorial plate and the metaphase localization in the plate of the chromosomes are just like the anaphase movement due to a specific activity of the centromeres, as evidenced in organisms with localized centromeres by the fact that only these chromosome parts show a strict localization in the equatorial plane at metaphase while the chromosome arms often hang out of the plate in various directions. So if we accept this opinion of a difference in the mechanism, we would obviously have to ascribe to the centromere two quite different kinds of specific activity. It should be noticed, however, that the transverse equilibrium at metaphase and the positions of trivalents and T-chromosomes demonstrate that the forces operating on the centromeres at *metaphase* (*cf.* pp. 445—446) are of such a type that they would be capable of accounting also for the *prometaphase* movements to the plate and for the *anaphase* movements to the poles. This means that we have to accept the existence of a mechanism *capable* of performing the anaphase movement and which works without continuous traction fibrils. Under such circumstances it certainly appears as a quite *unwarranted assumption* to suggest that this mechanism works only until the end of metaphase after which it is replaced by quite a different mechanism. There is also good support for the idea that the metaphase equilibrium and the poleward anaphase [1] movements are caused by the same mechanism from a study of the events taking place during the first division of meiosis in organisms having a mixture of paired and unpaired chromosomes. Here it is found that some chromosomes perform anaphase movement while others in the same cell and at the same time obviously are in a metaphase stage.

[1] There is another movement besides the poleward one which also takes place at anaphase in many organisms, *viz.* a separation of the daughter chromosomes due to a stretching of the spindle as a whole (Ris, 1943, 1949). This stretching does not move the chromosomes relative to the poles, and may be disregarded in the present discussion. Such a stretching is negligible or absent in *Luzula* as in many other higher plants.

What ideas would then appear probable concerning the physical organization of the spindle and the mechanism producing the pull on the chromosomes? It has been suggested by some adherents to the simple traction fibre idea that there is a contraction of the traction fibres by a folding up of their protein polypeptide chains (*e. g.*, SCHMIDT, 1939). If this theory shall be formulated in a way giving agreement with the prometaphase and metaphase behaviour of the chromosomes, we must assume that there is a continuous tendency of the traction fibre to fold up in this way all during the division, thus making it exert a kind of elastic pull on the chromosome. I cannot possibly see how such an idea or any similar hypothesis could allow of an easy passage of the chromosome body through the traction fibre leaving it functionable after this process. Another difficulty in this idea is that it leads to the assumption that at prometaphase, when the two traction fibres are synthesized, one of the two constitutionally identical fibres is synthesized in an extended state and the other one is synthesized in a contracted state, depending on the asymmetric position of the chromosome outside the equator.

In the spindle there is no doubt a definite spatial arrangement of its component micelles or molecules. Its fibrillated appearance in fixed preparations and its double refraction in the living as well as in the fixed state (*e. g.*, SCHMIDT, 1939) demonstrate that it contains fibrous particles in parallel arrangement. Such results indicate that the spindle is either in a solid state or in a liquid crystalline one. (The theoretical alternative of double refraction of flow in an ordinary liquid is scarcely applicable to the spindle of mitosis.) In the former case the position of the particles is a static one, in the second case the spindle represents a system of particles in dynamic equilibrium, the particles continuously exchanging position with one another as in a liquid, but still preserving their characteristic parallel orientation. Droplets of liquid crystalline type can be found in certain colloidal solutions such as those of vanadium pentoxide; such droplets are called tactoids and are often spindle shaped. If the spindle of mitosis were a solid it would make a fairly high resistance to the movements of the chromosomes within it and such movements would lead to a destruction of its structure in a manner which is not spontaneously reversible. In a tactoid, on the other hand, it is an easy matter for bodies to move around, and the movements would only lead to a momentarily upset of the equilibrium which is restored spontaneously after the passage of the body.

The idea that the spindle of mitosis is a tactoid has been put forward by FREUNDLICH (1927, 1937), FREUNDLICH, ENSLIN and SÖLLNER (1933), and PFEIFFER (1938). A more detailed hypothesis of this kind also striving to explain the movements of the chromosomes was presented by BERNAL (1940). BARBER and CALLAN (1943) interpret their results in terms of BERNAL's theory and supplement it by some ideas of their own. The hypothesis of BERNAL cannot be maintained in its details, but the general idea of the tactoid nature of the spindle may well turn out to be quite fertile. (It may be mentioned in passing that DARLINGTON, 1937, also uses the expression »liquid crystal» when he describes his ideas concerning the spindle, *l. c.*, p. 522.)

The fact that the chromosomes are able to move transversely across the spindle fibres and do so normally during the course of mitosis, strongly argues that the spindle is of tactoid nature. This would, according to the present results, be the case not only for the general ground substance of the spindle but also for that fibrous structure which is attached to the chromosomes (the chromosomal spindle fibres) and which may be suspected to play an essential rôle in the chromosome movements.

It is easy to get a general idea of how forces working on bodies may arise in a tactoid, but it may scarcely be possible to predict in detail concerning these forces. Thus, it is clear that any foreign body located inside the tactoid will by its mere presence disturb the spontaneous equilibrium of the particles and when the system strives to restore that equilibrium there will result forces acting on the body. It was suggested by ÖSTERGREN (1945 b) that forces of this type were involved in the tendency of the spindle to extrude the chromosome arms out of it, which is manifested in the transverse equilibrium of the chromosomes on the metaphase plate. Another force that acts in the same direction on the chromosome arms may simply be the surface tension of the spindle tactoid (ÖSTERGREN, 1949).

Now, the forces acting on the centromeres are of a specific type quite different from those acting on chromosome arms and nucleoli. If these centromere forces also arise from a disturbance of the equilibrium of the spindle tactoid, it is clear that this disturbance caused by the centromeres must be of a quite specific kind. One may suggest that the chromosomal spindle components that are found to attach to the centromeres may be the result of such a specific disarrangement which the centromeres provoke in the equilibrium of the spindle particles. The attachment of the chromosomal spindle components to the centromeres may be interpreted as the result of a tendency of the tactoid particles to set perpendicularly to a special surface of the centromere

with their ends attached to this surface. By taking up this position they are brought in a state of closer packing and a higher degree of orientation than in their spontaneous arrangement, an opinion suggested by the actual appearance of the chromosomal spindle fibres. This disarrangement of the particles at the centromere influences other particles in their neighbourhood so there results a secondary disarrangement of them to give a region of disarranged particles extending from the centromere towards the poles. This is no doubt a highly hypothetical point, but the appearance of the chromosomal spindle components does suggest that the organizing influence producing them is located at the centromere, the chromosomal spindle components are much more dense and distinct there and grow gradually more indistinct towards the poles (*e. g.*, ÖSTERGREN and PRAKKEN, 1946; SCHRADER, 1944) as expected on the basis of the present idea. This region (the chromosomal spindle component) will naturally, as must be assumed in the case of a tactoid, be in a dynamic equilibrium with the rest of the spindle, continuously exchanging particles with it. It is likely that such a region will strive to shorten spontaneously as that would lead to a reduction of the number of rearranged tactoid particles; this would then be the origin of the pull which the spindle exerts on the chromosomes. When such a »traction fibre» shortens it will lose particles to the rest of the spindle and when it lengthens, as it will do when pulled at by a force stronger than its own, it will gain particles from the rest of the spindle. One might object to these ideas on the ground that it is difficult to conceive how a structure that is fairly liquid in nature can exert a pull of any noticeable degree without breaking into pieces itself. This objection is disposed of by making reference to the phenomenon of surface tension in which it is demonstrated that a surface film of liquid nature is capable of pulling at bodies with quite strong forces. The effect assumed above to work in the spindle of mitosis is indeed of a type quite closely related to surface tension. The surface of a liquid is also a region in dynamic equilibrium with the rest of the liquid and it is also striving to be decreased as much as possible by giving up substance to the rest of the liquid, thereby exerting a pull on bodies preventing its contraction.

In the case of hypotheses assuming that the chromosomes are pulled at by contractile fibrils it is necessary to assume that the fibril is fixed with its other end at a definite point capable of carrying the load of the pull. In the present case there is no need for that assumption; naturally here, too, something must carry the load of the pull that is

exerted on the chromosome, but this need not be localized in a definite point, the force may be distributed over a large region of the spindle.

I wish to emphasize that the present hypothesis does not pretend to be based on any intimate knowledge of the properties and possibilities of tactoids. To judge from the literature, it would seem that the tactoids have not yet been sufficiently investigated to allow us to make safe conclusions concerning their possibilities in supplying detailed solutions to such biological problems as the present ones. My hypothesis is based chiefly on biological data such as the freedom of mobility of the chromosomes in the spindle, the appearance of the chromosomal spindle fibres, *etc.* This hypothesis may rather be regarded as an interpretation of the events of mitosis than an explanation of them.[1]

A summary of the present hypothesis was published in the paper of ÖSTERGREN and PRAKKEN (1946).

Further support for the tactoid idea of the mechanism for the chromosome movements is supplied by the process of co-orientation occurring during the prometaphase movements at the first division of meiosis in most organisms. The co-orientation results from a mutual influence of the partners of bivalents and multivalents of such a kind that neighbouring chromosomes orient themselves towards opposite poles. Thus, it is found in bivalents that the two partners always orient towards opposite poles, and in complex rings such as in *Oenothera* and *Rhoeo* there is a preference for a regular zig-zag arrangement. How does it occur that the two partners of a bivalent only send their »traction fibres» towards opposite poles, and not just as often to the same pole, and why do the paired chromosomes not lie in an indifferent position as univalents usually do on the same spindle? To explain that we will probably have to ascribe to the centromeres an ability to turn around in the spindle, changing their orientation until an equilibrium is reached in the co-oriented state. Such a turning around is easily conceivable in a dynamic system of tactoid type, while it is, as far as I can see, irreconcilable with an attachment of the centromeres to contractile fibrils of the solid type. On the basis of the tactoid hypothesis presented above, I have

[1] It may be pointed out that the constitution ascribed to the »chromosomal spindle fibres» in the present hypothesis implies that they are of »positive tactoid» structure like the rest of the spindle. Thus they must not be confused with the negative tactoids in the hypothesis of BERNAL (1940), which have a quite different constitution and also another localization in the spindle. SCHRADER (1947) has criticized the opinion that the chromosomal spindle fibres would be negative tactoids. This opinion has not been suggested by anyone, however, not even by BERNAL (1940) or BARBER and CALLAN (1943), as believed by SCHRADER.

developed a simple explanation of the phenomena of co-orientation, applicable even for such complicated cases as the *Oenothera* rings. This hypothesis will appear elsewhere.

Naturally, the present tactoid hypothesis can also be regarded as a kind of traction fibre idea, it may be said to operate with traction fibres of tactoid nature, shortening or lengthening by, respectively, losing or gaining substance to or from the rest of the spindle. It should be reconcilable with the arguments given for the traction fibre principle by CORNMAN (1944).

I am not willing to subscribe to the speculations of PIZA (1943) on »the uselessness of the spindle fibres for moving the chromosomes», but on the other hand his paper deserves to be mentioned for some good ideas in it. Thus, PIZA finds that the chromosomes of the scorpion *Tityus* are attached to the spindle during the prometaphase movements long before they reach the equatorial plate. He finds that »in spite of having both extremities connected with the poles by means of spindle fibres, these chromosomes can move and rotate freely in order to assume their ultimate metaphase shape». Although he interprets his results on the basis of the idea that these chromosomes have a centromere in each end, which is probably a mistake, the observation does not grow less significant if we instead assume that the chromosomes have a diffuse centromere, as may be more probable. This observation would be in good agreement with the results and hypotheses given in the present paper.

SUMMARY.

Mitosis was studied in the root tips and pollen grains of *Luzula purpurea*. The chromosomes of this species have a diffuse centromere, as previously reported by MALHEIROS, CASTRO and CAMARA. It often occurs that one chromosome is lying across another in the metaphase plates. Such cases imply an interlocking of chromosomes and spindle fibres in a way that might be expected to cause serious troubles at the anaphase separation. As no such anaphase disturbances occur it is concluded that the chromosomes move transversely across the spindle fibres and even through the chromosomal spindle fibres, which may be assumed to be the active agents at the mitotic movements. This is evidence against the simple traction fibre hypothesis working with contractile fibrils and it suggests a tactoid nature of the spindle. A hypothesis to explain the chromosome movements is suggested that involves »traction fibres» of tactoid nature shortening or elongating by losing

substance to or gaining it from the rest of the spindle. Hypotheses concerning the organization of the diffuse centromere are also put forward.

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RADIOPHOSPHORUS, SEEDLING LETHALITY AND CHROMOSOME DISTURBANCES

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OWING to the steadily increasing use of radioactive tracers in physiology and medicine it has become a pertinent object to examine their cytogenetical effects, too. Since normal phosphorus (P31) forms an essential constituent of the genes and chromosomes and its radioactive isotope (P32) is easily available and inexpensive, we decided some time ago to include it in chromosome and mutation work, adding it to the chemicals, X-rays, ultraviolet and neutron radiations, the effects of which are now being investigated at Svalöf. P32 completes the previous studies of ionizing radiations, since it breaks down under the formation of β-particles only, no α-particles or γ-rays being produced. The electrons have a weak penetration and consequently give a more localized effect than X-rays and neutrons. If P32 is forced to enter the nucleus and to form part of the chromosome proteins and nucleotides, its continuous local action may induce definite mutations of interest to geneticists and plant-breeders. In addition to these studies we have begun to examine the distribution of radiophosphorus in different tissues and cell constituents. In this way we hope to throw some light on the metabolic interactions of cytoplasm and nucleus. One of the authors (EHRENBERG) will specially deal with the cytoplasmic effects relating to permeability and ion exchange.

Simultaneously with the work reported in this paper EHRENBERG and GUSTAFSSON carried out a series of experiments using P32 in mutation studies bearing upon fruit-trees. The results will be described on a later occasion in collaboration with the staff of the Fruit-Tree Breeding Institute at Balsgård (Director: Dr. I. GRANHALL).

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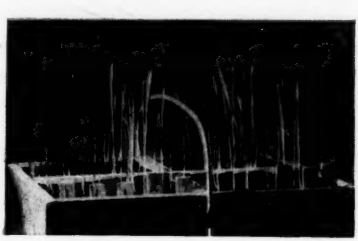
MATERIAL AND METHODS.

The radioactive phosphorus used in this investigation is the common one (P32) obtained in the form of phosphate ions from the Atomic Energy Commission of the U. S. A. through the agency of the Swedish Isotope Committee. Although other substances are also present in the phosphorus solutions (*cf.* HEVESY, 1948, p. 22) it is by now settled that the retardation and inhibition of growth, reported in this paper, is due to the radioactive substance itself. However, in order to prove this for certain we had to arrange for special experiments. In a recent article by THOMAS and NICHOLAS (1949) it was shown that after neutron bombardment of disodium hydrogen phosphate [$Na_2HP(31)O_4$] about half the amount of radiophosphorus that has arisen is in the form of phosphite. This ion works in quite another manner than a phosphate, since it is not actively taken up by the roots. Thus caution is required in the interpretation of the results in radioactive tracer procedure when P32 obtained by bombardment of a phosphate is used. The radiophosphorus, used by us, was obtained from sulphur.

Cereal seed of two-rowed barley, hexaploid spring wheat (*Triticum vulgare*) and tetraploid wheat (*Triticum dicoccum*) were included in the analysis. Unfortunately the available seed material of the diploid *Triticum monococcum* had been so severely damaged by the threshing that its germination capacity was almost nil. Dry, dormant seeds were set to germinate for 72 hours in small Petri dishes containing known amounts of radioactive solutions. After a careful washing the germinating seeds were placed in small paper boxes with soil, according to a method worked out for silviculture by NILS BERNER, forester (see »Skogen», 1949, p. 74, for a full description), and the different series were placed at random in large wooden boxes (Figs. 1—2). Measurements of growth were performed at distinct intervals. Whole seeds and seedlings or, as the case may be, separated stem, root and endosperm parts were analysed with regard to their contents of P32. Unless otherwise stated the determination of P32 is founded on five random seeds, seedlings or parts of seedlings in each special case. This number may be regarded as rather low and now and then causes slight irregularities owing to differences in seed and seedling development. However, this is taken into account in the discussion of the results. All series include one or two controls.

The samples of plant material were oxidized in H_2SO_4—HNO_2 mixtures. After adding 37,5 mg. of $Na_2HPO_4.2H_2O$ to each sample as



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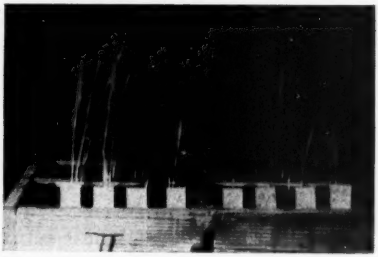

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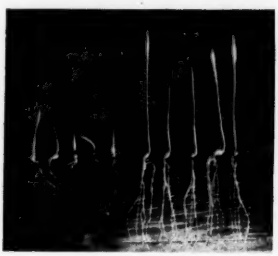

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Fig. 1. Growth of barley seedlings after treatment with P32 of variable strength (3 days in solution, 7 days in soil). From left to right: 1 — 20 μC/seed, 2 — 2 μC/seed, 5 and 6 — controls. — Fig. 2. From left to right (two rows of each series): control, 15 μC/seed, 25 μC/seed, 5 μC/seed. — Fig. 3. The influence of P32 (20 μC/seed) on root formation and growth. Control seedlings to the right. — Fig. 4. Radioautograph of wheat (20 μC/seed for 3 days), showing the concentration of P32 into the meristematic zone of the root tips (sections 15 μ thick).

carrier, the radiophosphorus was precipitated as magnesium-ammonium phosphate. The methods of KOLTHOFF and SANDELL (1943, p. 395) were applied. The precipitates were collected on aluminium dishes (*cf.* HEVESY, 1948, p. 45), and the radioactivity was measured by means of a Geiger-Müller counting tube (HEVESY, *l. c.*, p. 57 ff.). The accuracy of the measurements was about 10 %.

For details relating to the *Allium* test, see p. 482 and LEVAN, 1949.

SEEDLING LETHALITY.

These studies ultimately aim at the application of P32 in mutation work involving agricultural plants. Barley seeds were selected as chief material. In the *first* experimental series forty seeds were given 20, 2, 0,2 and 0,02 μC per seed for 72 hours in 4,5 ml. solution. After 24 hours

4,5 more ml. of water were added to the solution. After planting the seeds and seedlings their growth was measured at distinct intervals. The data run as follows (*cf.* also Fig. 5). In this experiment the measurements were performed 15 days after the onset of germination.

	20	2	0,2	0,02 μC/seed	Control 1	Control 2
Seedling growth in mm.	27,6	173,4	177,8	172,2	182,8	165,7

The roots of the highest concentration remained short and stunted and produced small tumour-like formations (Fig. 3). The leaves hardly pierced the coleoptiles. Evidently 20 μC/seed implied a lethal dose. whereas 2 μC/seed gave an almost normal growth and development.

In the *second* set of experiments forty seeds were given 25, 15 and 5 μC/seed for 72 hours in 5 ml. of solution. After 48 hours two more milliliters of water were added. The two first concentrations proved lethal. The following survey shows the growth of the seedlings after 11 days (3 days of treatment, 8 days of cultivation in soil).

	25	15	5 μC/seed	Control
Seedling growth in mm.	18,0	29,4	78,1	118,9

The data indicate that even 5 μC/seed are effective in retarding growth. After the planting, some roots of this series gave small tumours which were pierced by the growing root tips.

In a *third* set of experiments forty seeds of barley as well as of hexaploid and tetraploid wheat were given 20, 15, 10 and 5 μC per seed for 72 hours in 5 ml. of solution. As previously mentioned, seeds of *Triticum monococcum* were also included, but, since they had been damaged by the threshing, merely a few were alive. This species will be left out of discussion. After washing and planting the seeds and seedlings, growth after 13 days was as follows.

	20	15	10	5 μC/seed	Control
Barley	18,3	23,4	55,5	145,7	194,2 mm.
Triticum vulgare	20,9	20,7	25,5	38,2	215,4 »
Triticum dicoccum	21,5	24,2	27,0	26,8	190,9 »

Concentrations of 15 and 20 μC/seed inhibited the growth of barley seedlings completely. With 10 μC/seed the result was less pronounced. A treatment with 5 μC/seed, finally, gave but a weak response. After the planting in soil small tumours were detected in all concentrations.

The two wheat species are obviously more susceptible to P32 than

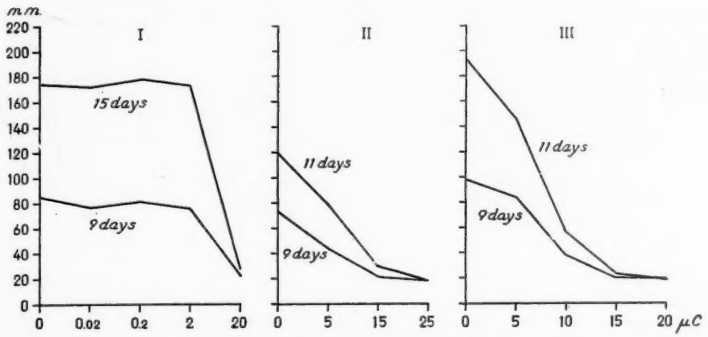

Fig. 5. Growth curves of barley seedlings; treatment of dormant seeds with P32 for three days. On the x-axis the amount of radiophosphorus (μC/seed), on the y-axis the growth (in mm.).

is barley. In fact, all concentrations, including the lowest one, proved lethal in *Triticum dicoccum*, possibly less so in *Triticum vulgare*, where a slight growth was detected in the low concentration of 5 μC/seed. The results were checked by one more week's study of the planted seedlings. These largely remained in the coleoptile stage.

To decide definitely that the inhibition of growth cannot be due to any by-products (*cf.* p. 470), present in the P32-solution delivered, a *fourth* experiment was carried out. On May 6th fresh solutions containing 20 and 5 μC/seed were compared as to growth inhibition with a solution kept since March 28th. A set of seeds was given a treatment that forty days previously corresponded to 20 μC/seed but that on May 6th, owing to the decay of P32, implied but 3μC/seed. The following

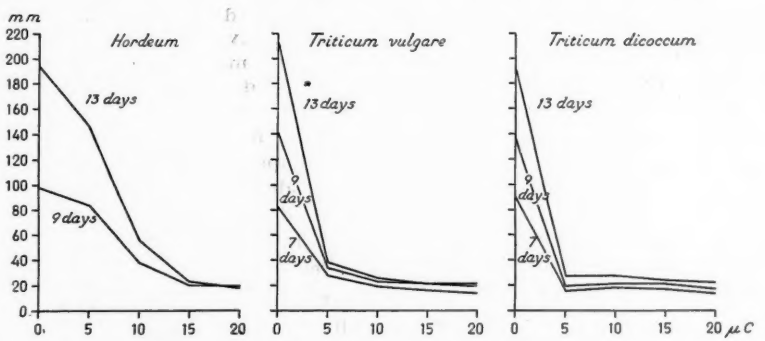

Fig. 6. Growth curves of barley, hexaploid and tetraploid wheat in parallel experiments.

figures, obtained after 84 hours' treatment with P32 and five days' subsequent cultivation in soil, indicate that the effect of the old solution actually corresponds to less than 5 μC/seed.

	Fresh solutions		Old solution	Control 1	Control 2
	20	5 μC/seed	»20» = *ca.* 3 μC/seed		
Seedling growth in mm.	12,9	38,3	57,2	115,6	108,1

The rate of cytological disturbances produced by the old solution answered to the effective amount of P32 still present (p. 480 ff.).

Under the conditions prevailing in these experiments (room temperature about 20° C.) barley seedlings cannot develop after treatments with more than 10 μC/seed (forty seeds in 4,5 ml. of solution). Wheat is much more susceptible, even 5 μC/seed are completely lethal to *Triticum dicoccum* and almost lethal to *Triticum vulgare*. This difference in susceptibility depends on certain biochemical properties, which will be elucidated in the next section.

The lethal effects of P32 have been studied in field experiments, too. One of the authors (GUSTAFSSON) has carried out a series of treatments in collaboration with Mr. J. MAC KEY. The concentrations ranged from 0,6 to 10 μC/seed. After the treatment, which lasted for 96 hours, the seeds and seedlings were planted directly in the experimental fields.

Concentrations of 0,6 to 2,5 μC/seed gave normal-looking seedlings, with but a slight decrease in germination capacity. After fourteen days concentrations of 5 and 7,5 μC/seed had produced about 70 % seedlings, a great number of which subsequently died. The concentration of 10 μC/seed, finally, produced less than half the control number of seedlings, many of which were very weak and died. The radioactive effects are less pronounced in the field experiments than indoors in the laboratory. The cause is no doubt that the germination of the field material proceeded at a lower average room temperature (17 instead of 20° C.). In consequence, the radiophosphorus was absorbed to a less degree, a fact that was subsequently revealed by electron counting.

THE DISTRIBUTION OF RADIOPHOSPHORUS.

Four different biochemical problems have so far been examined: (1) How much of the P32 present in the various solutions is absorbed by the germinating seeds? (2) How much of the absorbed phosphorus goes into the stem and root parts, respectively? (3) How much of the

absorbed radiophosphorus — its decay being ignored — remains in the plant for a definite time after germination? (4) Is there any difference in phosphorus intake, corresponding to the difference in susceptibility, between barley on one hand and the wheat species on the other?

The first problem may be answered to the effect that germinating seeds absorb higher amounts of radiophosphorus the more diluted the surrounding medium. Although the various series, of course, show differences in absorption, this rule consistently holds true (Table 1).

TABLE 1. *Amount of radiophosphorus absorbed.*

	0,02	0,2	0,6	1,3	2,0	2,5	5,0	7,5	10	15	20 μC/seed
Laboratory exp. (72 hours)											
Barley I	92 %	62 %			57 %						50 %
» II							54 %		47 %	33 %	28 »
Hexaploid wheat							66 »		64 »	71 »	46 »
Tetraploid wheat							70 »		73 »	68 »	43 »
Field exp. (96 hours)											
Barley			48 %	43 %		30 %	21 »	20 %	20 »		

In addition, the figures of Table 1 show that the wheat species absorb more P32 than does barley in the same set of experiments (*cf.* barley II with hexaploid and tetraploid wheat). Since wheat seeds weigh considerably less than barley seeds, this fact implies that the amount of P32, calculated per gram fresh weight, is already from the beginning about two or three times higher in wheat than in barley.

The ion exchange involving P32 was determined by measuring the contents of P32 at the time of planting (72 hours after the onset of germination) and then after one and after two weeks. All values obtained were recalculated to the beginning of the experiments. By this recalculation all figures become directly comparable, irrespective of the decay of P32 (half-life 14,3 days). An experiment in barley will be specially considered here. The wheat species produce similar results.

TABLE 2. *Absorption of P32.*

Given dose	At the time of planting	%	Seven days after planting	%	Fourteen days after planting	%
5 μC/seed	2,7 μC	54	1,9 μC	39	0,8 μC	16
10 »	4,7 »	47	2,9 »	29	1,9 »	19
15 »	4,9 »	33	3,2 »	21	3,0 »	20
20 »	5,5 »	28	7,1 »	35	4,9 »	25

Both in barley and wheat the relative intake of radiophosphorus is higher the lower the concentration of the surrounding medium (*v.* above). Simultaneously the ion exchange is increased. This is clearly indicated by the fact that at the highest and most lethal dosage (20 μC/seed) the amount of P32 remains constant over a long period of time. Possibly the explanation is quite simply that, with the heavy cell damages following upon high radioactive concentrations, the metabolic functions are so arrested that no active exchange can take place.

TABLE 3. *The effects of increasing amounts of normal phosphate on the absorption of P32 and the seedling growth.*

Amount of P 32 per seed	Total amount of phosphate in μmol per 5 ml. of solution						Non active or inactive phosphorus present
	0,068	4 × 0,068	16 × 0,068	64 × 0,068	256 × 0,068	1024 × 0,068	
1,25 μC							
Absorption	*90 %*	69 %	55 %	45 %	39 %	30 %	
Growth	*128* mm.	137 mm.	120 mm.	126 mm.	135 mm.	146 mm.	
5 μC							
Absorption		*50 %*	(81 %)	45 %	23 %	20 %	
Growth		*82* mm.	(77 mm.)	82 mm.	97 mm.	118 mm.	
20 μC							
Absorption			*42 %*	35 %	26 %		
Growth			*13* mm.	13 mm.	17 mm.		
Control I							
Growth			139 mm.	138 mm.	151 mm.	133 mm.	
Control II							
Growth							*156* mm.

Figures in italics indicate that no inactive phosphorus was added.

A *fifth* set of experiments was performed in order to make clear the influence of (1) radioactivity and (2) normal phosphate upon the intake of P32. As indicated by Table 3, the seeds were given 1,25, 5 and 20 μC (calculated per seed and 5 ml. of solution). Inactive phosphorus (Na_2HPO_4) was added, increasing the total concentration of phosphorus by 4, 16, 64, 256 and 1024 times. Two controls were included, one without any phosphorus, either active or inactive, one with increasing concentrations of inactive phosphorus. The growth of seedlings was measured nine days after the beginning of experiments (five days after the time of planting). The figures of phosphorus absorption rest upon two different electron determinations, each involving five seeds or seedlings.

The figures in italics of the table indicate the effects of P32 without any inactive phosphorus added. The result is similar to that re-

ported previously. Twenty μC/seed act completely lethalizing, 5 μC are less effective and 1,25 μC even less so. With the increase in radioactivity there is a change in the absorption of P32, making it decrease from 90 % with 1,25 μC to no more than 42 % with 20 μC. None the less, the absolute amount of radiophosphorus absorbed rises considerably. This causes a marked inhibition of growth.

As might be foreseen, the absorption of radioactive phosphorus is influenced by the amount of normal phosphorus present in the solution. With 1,25 μC/seed it falls from 90 % to 30 % in the highest concentration, with 5 μC from 50 % to 20 %, and with 20 μC from 42 % to 26 %. Answering to this decrease in absorption the growth becomes less retarded. Consequently we can state that the absorption of radiophosphorus depends (1) on the intensity of radioactivity, (2) on the concentration of inactive phosphorus present.

The important question what parts of the plant primarily store the radiophosphorus must be considered in some detail (Table 4). The plants were divided into green parts (»stem»), roots, as well as the remainder of the seed (endosperm, in barley also lemma and palea, *etc.*). Here it must be mentioned that the distribution of P32 has previously been studied by the help of autoradiographs (SCOTT RUSSEL and co-workers, 1949). It concentrates into distinct tissues. Important for mutational problems is the accumulation of P32 in apical meristems of the stem, leaf primordia, the bases of the younger leaf sheaths and the initials of adventitious roots. Using ILFORD's nuclear research emulsions of 40 μ thickness, the present authors prepared some autoradiographs of wheat and barley roots and found a conspicuous concentration of P32 into the extreme meristematic zone of the root tips (Fig. 4).

First barley: In all concentrations plants, seven days of age, store a large amount of radiophosphorus in the seed remainder. After another week of cultivation the contents are but slightly altered. In the two lowest concentrations stem and root parts contain small amounts of P32. With high concentrations the roots contain more P32. Especially striking are the high amounts absorbed in solutions with 15 and 20 μC/seed. Owing to ion exchange roots lose most of their radioactivity in one week. From a mutational point of view it is worth mentioning that with 5 μC, which is but slightly lethal, the green parts lose their radioactivity rapidly. With 10 μC (and onwards) they keep it longer. For this reason dosages of 7—8 μC/seed ought to give the most satisfactory results in field work — at least under environmental conditions similar to those present here.

TABLE 4. *Absolute amounts in μC per seed and plant of radiophosphorus absorbed.*

Dose		Barley			Hexaploid wheat			Tetraploid wheat		
		At the end of treatment	One week after end of treatment	Two weeks after end of treatment	At the end of treatment	One week after end of treatment	Two weeks after end of treatment	At the end of treatment	One week after end of treatment	Two weeks after end of treatment
5 μC	»Stem»		0,48	0,08		1,20	1,85		1,18	1,07
	Roots		0,17	0,03		0,53	0,42		0,76	0,21
	Remainder		1,28	0,71		0,66	1,50		1,31	0,75
	Sum	2,72	1,93	0,82	3,30	2,44	3,00	3,48	2,64	2,00
10 μC	»Stem»		0,18	0,22		1,20	2,40		2,65	2,14
	Roots		0,07	0,10		0,51	0,29		0,41	0,19
	Remainder .. .		2,35	1,75		1,76	1,25		1,23	1,30
	Sum	4,66	2,85	1,94	6,44	3,44	3,94	7,34	4,10	3,62
15 μC	»Stem»		0,34	0,30		1,86	3,64		2,90	2,36
	Roots		0,19	0,10		0,80	0,72		1,00	0,60
	Remainder		2,68	2,57		1,98	2,16		3,50	3,14
	Sum	4,94	3,20	2,98	10,60	4,64	6,50	10,26	7,65	6,10
20 μC	»Stem»		1,85	0,52		1,02	5,13		(4,6)	4,50
	Roots		0,58	0,05		0,50	0,80		—	0, 92
	Remainder		4,68	4,34		2,42	2,90		(6,5)	3,53
	Sum	5,52	7,10	4,90	9,24	3,94	8,83	8,60	(11,1)	8,97

The wheat species behave in an entirely different manner. As mentioned previously, the total phosphorus intake of wheat kernels is two or three times as high as in barley, calculated per gram fresh weight. The high amounts of radiophosphorus stored in roots and stems are especially remarkable. The lethalizing treatment and its consequent injuries to cell metabolism impede the decrease in P32 content even after a second week. This state of things is accentuated by the fact that assimilation is not proceeding. Consequently the plant weight cannot rise; rather does it decrease, in this way automatically augmenting the radiophosphorus. The data indicate that the wheat species store more P32 in root and stem parts and keep it there longer than does barley. If this holds true also for non-lethal dosages, wheat ought to be considered a superior species for mutation experiments involving radiophosphorus.

The facts discussed above afford an explanation why wheat species are more susceptible to P32 than barley. Firstly, the wheat species absorb two or three times as much radiophosphorus in three days (calculated per gram fresh weight). Secondly, the root and stem parts of wheat species receive and store higher amounts of P32, with a proportionally less amount in the seed remainder. In barley, on the contrary, the seed remainder contains most of the radioactive substances absorbed, in contrast to root and stem parts, which possess small amounts that disappear more or less rapidly. Until further investigations have been carried out there is no reason to enter into detailed discussions. Suffice it to say that wheat species and barley apparently regulate their phosphorus absorption and storage in different manners, at least in the concentration ranges dealt with in this paper. There is no special sensitivity characteristic of the wheat cell in contrast to the barley cell. Growth inhibition and chromosome disturbances directly correspond to the amount of radiophosphorus absorbed.

Full lethality appeared in all the cereal species used when the germinating seeds had taken up an amount of radiophosphorus that corresponds to about 60 μC per gram fresh weight. One week after the planting, when the lethality can be observed for certain, the roots of fully lethal plants and series contained 40 μC/gram fresh weight or more. Simultaneously fully lethal »stem parts» showed contents of about 30 μC or more. These figures, which must be regarded with due caution, indicate the following P32-concentrations to be completely lethalizing:

in whole seeds ..... $\geqq$ 180 μC . day/gram fresh weight
roots ..... $\geqq$ 400 » » »
stem parts ..... $\geqq$ 300 » » »

CHROMOSOME DISTURBANCES IN BARLEY AND WHEAT.

The frequencies and types of mutation induced by radiophosphorus will be analysed in progeny studies of material treated this year. However, in order to study the immediate chromosomal effect and by that the cytological back-ground of the mutation process, roots of germinating seeds of barley and wheat were fixed at regular intervals. Then the number of disturbed anaphases containing fragments and bridges was determined.

The radioactive solutions greatly delay division. This is due to the radioactivity itself, since the old solution, originally containing 20 μC/seed, gave numerous cell divisions after three days of germination, in contrast to fresh solutions with 20 and 15 μC/seed, where metaphases and anaphases were scarce or did not occur at all.

TABLE 5. *Number of cells with chromosome disturbances (barley).*

Dose	After 3 days of treatment	1 day in water after end of treatment	3 days in water after end of treatment	8 days in soil after end of treatment
20 μC/seed	No divisions	No divisions	$^{73}/_{77} = 95$ %	—
15 »	$^{6}/_{6} = 100$ %	»	No divisions	$^{12}/_{40} = 30$ %
10 »	No divisions	$^{31}/_{39} = 80$ %	$^{15}/_{17} = 88$ %	—
5 »	$^{58}/_{93} = 60$ %	No divisions	$^{52}/_{263} = 20$ %	$^{10}/_{107} = 9$ %
»20» = ca. 3 μC/seed	$^{36}/_{79} = 46$ %	—	—	—

The chromosomal effects are indeed very striking (Table 5). After three days of germination in P32-solutions of 5 μC/seed more than half the anaphases were abnormal. This corresponds in diploid cereals to an effect of at least 15000 or 20000 *r*-units (FRÖIER, GELIN and GUSTAFSSON, 1941; GELIN, 1941)[1]. After some days in water or soil there is a decrease in the amount of disturbances. The effect is, however, rather pronounced after three days in water, too. With 10—20 μC/seed it still corresponds to 25000 *r*-units or more, with 5 μC/seed at least to 10000 *r*.

[1] If we recalculate the P32 activity to roentgen units (according to SPINKS *et al*, 1948, and SCOTT RUSSEL and MARTIN, 1949), we find that the effectivities of β- and X-rays in causing cell damage and lethality are of the same order of magnitude. The present experiments do not permit any detailed analysis of this point.

TABLE 6. *Proportion of anaphase cells with chromosome bridges (barley).*

Dose	After 3 days of treatment	1 day in water after end of treatment	3 days in water after end of treatment	8 days in soil after end of treatment
20 μC/seed	—	—	$^{4}/_{73} = 5{,}5$ %	—
15 »	$^{0}/_{6} = 0$ %	—	—	$^{6}/_{12} = 50$ %
10 »	—	$^{1}/_{31} = 3{,}2$ %	$^{1}/_{16} = 6{,}3$ %	—
5 »	$^{2}/_{56} = 3{,}6$ %	—	$^{22}/_{52} = 42{,}3$ %	$^{7}/_{10} = 70$ %
»20» = ca. 3 μC/seed	$^{1}/_{36} = 2{,}8$ %	—	—	—
Sum	$^{3}/_{98} = 3{,}1$ %	$^{1}/_{31} = 3{,}2$ %	$^{27}/_{141} = 19{,}1$ %	$^{13}/_{22} = 59{,}1$ %

Cultivation of the seedlings in soil for eight days after the end of treatment still shows an appreciable amount of disturbances — for 15 μC/seed no less than 30 % and for 5 μC/seed 9 %.

Previously it was shown (p. 473) that the growth inhibition caused by the radioactive solutions is due to P32 itself, not to other substances present. The cytological effect of the old solution with originally 20 μC/seed (46 % disturbed anaphases) answers rather well to the calculated strength of the solution, about 3 μC/seed.

Of great interest are the proportions of the different disturbances in barley. Immediately after the end of treatment, as well as one day afterwards, almost all of the cytological disturbances consist of fragments (Table 6). Very few bridges are formed, in contrast to three days after the end of treatment and eight days of cultivation in soil. Reunions leading to two- and four-centromere formations are apparently scanty in barley seeds and seedlings until after a certain lapse of time. Bridges are more frequent, relatively seen, in low P32 concentrations. Since the roots of seeds given high concentrations of P32 keep their phosphorus for a long time, new breaks will continually appear and give rise to fragmented chromosomes. These are kept intact for some time. Consequently, bridges are rare immediately after the chromosome breakage has taken place. With low concentrations the absorbed P32 soon disappears. Thus no new breaks result and the old ones gradually reunite. In conclusion, the relative frequency of bridges will be higher the lower the original P32 concentration.

The high susceptibility of wheat seeds is reflected not only by increased disturbance frequencies but also by an increased number of fragments per disturbed cell. A treatment with 10 μC/seed produces about four fragments per disturbed anaphase in barley. In hexaploid wheat treated with 5 μC/seed there are no fewer than eight or nine

fragments per disturbed anaphase. The cell divisions are often highly aberrant. There is a tendency to higher bridge formation in wheat than in barley, but the evidence is not conclusive. Here it suffices to emphasize that hexaploid and tetraploid wheats produce higher rates of aberrations. This is certainly in part due to the polyploidy (FRÖIER, GELIN and GUSTAFSSON, 1941), but partially also to the higher intake of radiophosphorus.

RADIOPHOSPHORUS AND THE ALLIUM TEST.

The set of experiments to be reported here comprised two series of 17 concentrations, ranging from 10000 down to 0,05 μC per 100 ml. solution. Series 1 was begun on April 23 and included 10 concentrations from 50 to 0,05 μC/100 ml. Series 2 started on April 24 and comprised 7 concentrations from 10000 to 100 μC/100 ml. Tubes of 70 ml. in volume were used in series 1. In series 2 the tube volume was 10 ml.

The onion roots of series 1 were left in the radioactive solutions for four days and then moved into water. Series 2 was moved already after three days, since several roots showed signs of rotting. However, not even the highest concentrations were completely lethal, although growth was greatly retarded (Table 7).

TABLE 7. *Treatment and growth of Allium roots in P32-solution.*

Experiment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Concentration	10000	5000	2500	1000	500	250	100	50	25	10	5	2,5	1,0	0,5	0,25	0,10	0,05 μC/100 ml.
Length of roots:																	
3 (4) days of treatment, 1 day in water	30	45	35	37	33	45	47	50	55	76	62	71	83	65	60	60	53 mm.
3 (4) days of treatment, 3 days in water	30	45	33	42	52	56	72	73	76	82	76	98	104	65	76	73	67 mm.

No typical c-tumours occurred. In concentrations 4—6 (1000—250 μC/100 ml.) the root tips curved in different directions, a type of growth response that is not rare with treatments applying various metallic salts and relates to the c-tumour reaction. It may appropriately be called *the crochet-hook response.* After moving the onions into water this phenomenon immediately disappeared in concentration 6 (250 μC) and after three days in concentration 5 (500 μC). There is some indication that the medium concentrations 9—13 (between 25 and 1 μC) stimulate growth. However, this will be examined more carefully in

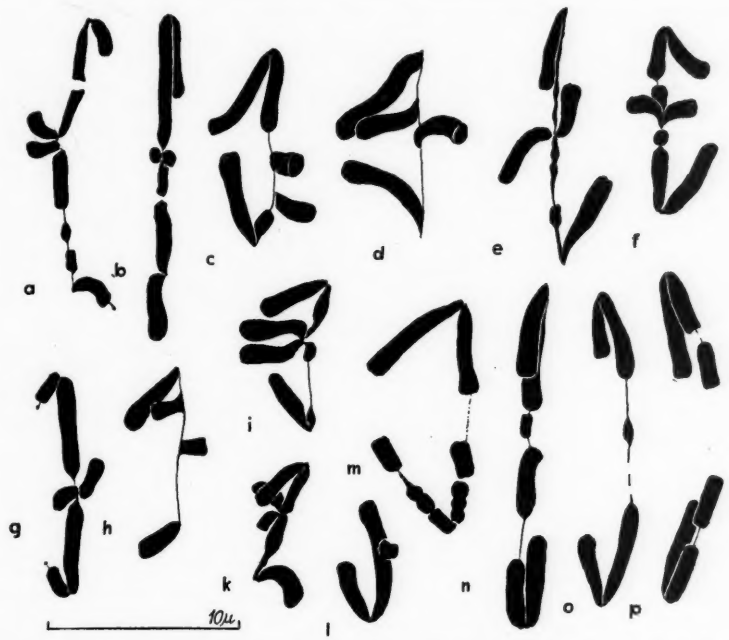

Fig. 7. Radiophosphorus and the *Allium* test. — *a*—*k*. Pseudochiasmata, in *a*, *b*, *e*, *f* combined with erosions. — *l*. Fragment attached sideways. — *m*—*p*. Erosions, in *p* a symmetrically attached fragment from a subsequent cell generation. — (*a*—*e*: 5000 μC/100 ml. for three hours; *f*, *i*—*n*: 2500 μC/100 ml. for three hours; *h*: 1000 μC/100 ml. for three hours; *g*: 25 μC/100 ml. for three days; *p*: 2,5 μC/100 ml. for three days.) — × 2900.

future experiments. A stimulating effect on seed germination, caused by radioactive thorium and uranium, was actually observed by BEVILOTTI (1944, 1945).

Roots were fixed after different intervals — 3, 24 and 72 hours of treatment, as well as after 24 more hours in water. The results may be summarized in the following way.

(1) *After 3 hours of treatment.* — All concentrations give divisions, although mitoses are less frequent in the highest ones. Even this short treatment produces chromosome disturbances. With 10000 μC some cells show greatly contracted chromosomes, in some instances simulating a weak c-mitotic effect, but no break-down of the spindle is found. Chromosome erosions (LEVAN, 1949), leading to secondary constrictions, as well as attached fragments, free fragments and pseudochiasmata are

abundant. A certain stickiness gives rise to bridges. With 5000 μC the effect is less toxic. Chromosome disturbances are frequent. No single division is entirely normal. Often more than ten fragments per cell are noticed. Several fragments resulted from pseudochiasmata (Fig. 7 *a—k*) but ordinary fragments occur, too. There seems to be a causal connection between erosions, free fragments and pseudochiasmata.

Concentration 3, with 2500 μC, gave similar results, but the number of disturbances was lower. In 100 cells there were numerous eroded chromosomes, 12 cases of pseudochiasmata, 3 cells with free fragments. In concentration 4, with 1000 μC, 100 cells contained several eroded chromosomes, three pseudochiasmata, one case of a free fragment and one chromosome bridge (possibly due to stickiness). In concentrations 5—9 (500—25 μC) occasional attached and free fragments were seen, but no pseudochiasmata and no distinct bridges. Below the concentration of 10 μC/100 ml. all mitoses appeared to be normal.

(2) *After 24 hours of treatment.* — In high concentrations there is a greatly reduced frequency of mitoses. Concentrations 1, 2, 4 were completely devoid of divisions, 3, 5 and 6 contained some single mitoses. Chromosome disturbances abound. The two commonest types are erosions — with their consequence: attached fragments — and free fragments, often in great numbers. Pseudochiasmata and bridges are less numerous. In concentration 5 (500 μC) all anaphases were abnormal. 50 cells of concentration 7 (100 μC) showed the following numbers of fragments:

Fragments	0	1	2	3	4	5
Cells	16	15	8	6	3	2,

i. e. fragments occur in 68 % of all cells. Almost every division contained one or more eroded chromosomes. One cell showed a chromosome bridge.

Concentration 8 (50 μC) contained almost no completely regular anaphases. Fifty cells had the following numbers of fragments:

Fragments	0	1	2	3
Cells	35	11	3	1,

i. e. the fragment frequency has decreased to 30 %. Seven cases of anaphase bridges were noticed. Two of them occurred in the same cell. One was a bridge between two chromosomes of the same anaphase group. In concentrations 9—11 (25—5 μC) solitary free and, more frequently, attached fragments were found. Rarely, chromosome bridges

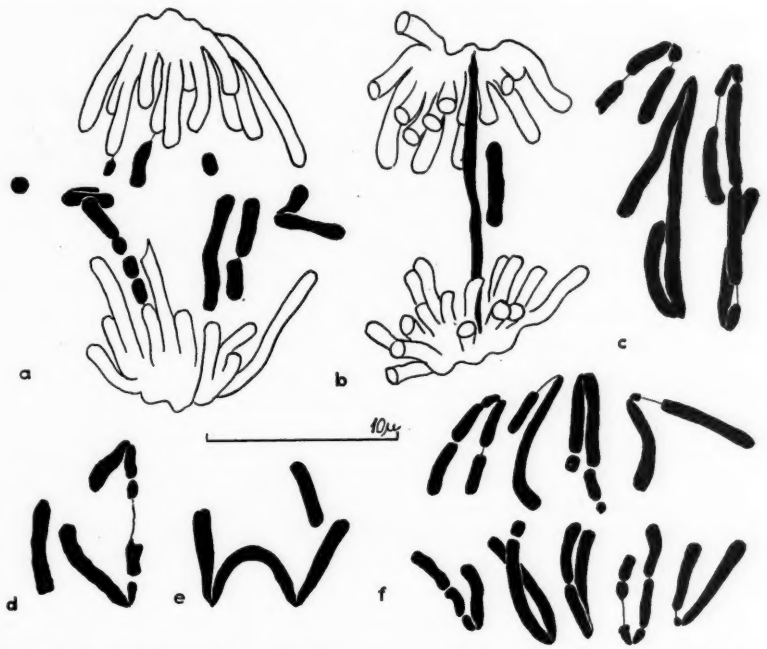

Fig. 8. Radiophosphorus and the *Allium* test. — *a*. Many fragments. — *b*. Bridge and one fragment. — *c*. Two bridges and one chromosome from the same cell, one bridge and the free chromosome eroded. — *d*. One eroded bridge and one fragment. — *e*. One bridge within one anaphase group and one fragment. — *f*. Nine anaphase chromosomes with erosions from one cell. (*a*: 2500 μC/100 ml. for 24 hours; *b*—*f*: 50 μC/100 ml. for 24 hours.) — × 2900.

appeared. From concentration 12 (2,5 μC) and downwards no disturbances were noticed.

(3) *After 72 hours of treatment.* — Concentrations 1—4 (10000—1000 μC) had no divisions. The meristematic tissues showed resting nuclei of a singular appearance — without or with very small nucleoli. (This was the case in wheat, too.) In concentrations 5—7 (500—100 μC) the disturbances had reached a considerable intensity, and numerous mitoses showed signs of a severe break-down. Thus there appeared in many cells highly contracted chromosomes, though other cells close by had very long and extended chromosomes. The chromosome erosion was at its maximum. Anaphases like the depicted ones (Fig. 9) were common. Here the chromosomes have broken down into small pieces. Many of these still join to chromosomes, but often they have loosened

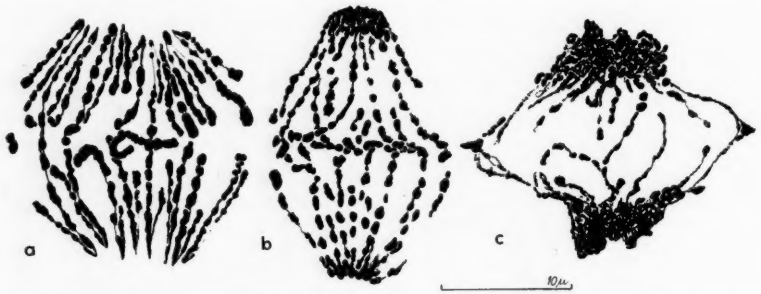

Fig. 9. Radiophosphorus and the *Allium* test. — Three cells subject to heavy erosion effects leading to cell lethality. (1000 μC/100 ml. for three days.) — × 2900.

from one another and form masses of free fragments, left at the equator.

The number of disturbances rapidly decreases with lower concentrations. Nos. 8 and 9 (50 and 25 μC) still contain numerous free fragments, pseudochiasmata and erosions. In concentration 10 (10 μC) cells with free fragments were found to the following extent:

Fragments	0	1	2	3
Cells	88	6	5	1,

i. e. to 12 per cent. In addition, there were three cases of pseudochiasmata and two cells with bridges. Also in concentrations 11 and 12 (5 and 2,5 μC) solitary disturbances occurred, but below these concentrations mitosis was apparently normal.

CONCLUSIONS.

In their paper of 1949 SCOTT RUSSEL and MARTIN arrive at the conclusion that the radiation damage effected by radiophosphorus has not been sufficiently realized in physiological research. We entirely agree upon this point and want to stress it even more. SPINKS, CUMMING and ARNASON (1948) regarded doses of 1,4 μC to be lethal to seeds of barley, wheat and sunflower, put for germination 48 hours previous to the treatment. When treating dormant seeds we found that doses of 5 μC per seed and more are completely lethalizing in tetraploid *Triticum dicoccum* and nearly so in the hexaploid *Triticum vulgare*. The cytological disturbances are extremely potent, answering to effects of 25000 *r*-units or more. Barley is less susceptible, it is true, but with a dose of

5 μC more than half of the cell divisions analysed contained chromosome disturbances.

In experiments applying the so-called *Allium* test it was found that even treatments with 2,5 μC in 100 ml. solution and lasting for three days gave chromosome fragmentation. With higher dosages there was an increasing amount of disturbances: pseudochiasmata, fragments, bridges due to stickiness (in the first three hours) or reunions and, finally, pronounced chromosome erosions (LEVAN, 1949), which lead to full lethality. Like MITCHELL (1947) we consequently warn against an indiscriminate use of radioactive tracers in medical therapy.[1]

Another finding of cytological significance implies that bridges due to chromosome breakage and reunion do not appear in barley roots immediately after the treatments. They were found first after a certain lapse of time. The reason for this is so far unknown. Nor do we know to what extent the result is valid also for other species. The physiological state of the broken chromosome surfaces may differ with the type and treatment of material.

The great susceptibility of wheat species contrasted with barley has found its biochemical explanation in the following facts. Germinating seeds of wheat species absorb two or three times as much P32 as does barley, calculated per gram fresh weight. In addition, the radiophosphorus is differently distributed within the young seedlings. Barley stores less of its absorbed radiophosphorus in stem and root parts, more in the seed remainder. In wheat, on the contrary, there is a considerable amount of P32 in stems and roots, but less so in the remainder of the seed. This implies, if the results do hold for non-lethal concentrations, that wheat species are superior objects when radiophosphorus is applied as the mutagenic agent. One week after the end of treatment there is, with 5 μC/seed, three times as much total radiophosphorus in stems of hexaploid wheat as in those of barley, after two weeks no less than twenty times as much. Calculated per gram fresh weight the proportions are even more displaced in favour of *vulgare* wheat.

We were able to make an estimation *per tissue* of the P32 concentrations which evoke a completely lethal condition. Provided that the

[1] Note, for instance, that certain diseases of the bone marrow (HALL, 1949) are treated with radiophosphorus in amounts of 5 to 35 millicuries, if given intravenously, or even more if administered orally with a consequent loss of 15 to 50 per cent through the urine and feces in the first six days. This implies doses more than thousand times as high as those that induce 100 % chromosome disturbances in cell divisions of germinating seeds.

phosphorus is acting over a 10-day period, the figures are: for whole seeds 18 μC per gram fresh weight, for roots 40 μC per gram fresh weight and for the green (»stem») parts 30 μC per gram fresh weight. As may be expected from a cytochemical point of view, autoradiographs prove that the absorbed radiophosphorus concentrates into the extreme meristematic zone of the root tips. However, the exact localization of the phosphorus *within* the cell is so far unclarified.

Although we know nothing about the mutations appearing in the next generation, their frequencies and types, we may conclude what treatments are appropriate for mutation experiments. The effects of a continuous manuring with P32 (or other tracers) or a continuous cultivation in radioactive solutions have not been established. Here it must be mentioned, however, that JACOBSON and OVERSTREET (1948) found tracers of Y, Ce, Zr, Cb, and Sr at activity levels of 0,1 μC per gram of soil to cause severe injuries in dwarf peas when acting over a 3-month period. With seed treatments that imply a superior method in X-ray and neutron work 5 to 10 μC of radiophosphorus, 25—40 % of which is absorbed, give a fair number of surviving plants in two-row barley, also in the field. The cytological irregularities produced by such doses are so drastic that less intense changes, involving simple chromosome rearrangements and gene mutations, are certainly frequent, too. Some will pass through cell divisions and eventually reach meiosis and the sex cells. In fact, ARNASON, CUMMING and SPINKS (1948) obtained such chromosome aberrations in tetraploid and hexaploid wheat by treatments of germinating seeds. They also found meiotic aberrations in *vulgare* wheat grown in soil to which a P32 fertilizer had been added. In their experiments, too, barley was less susceptible to P32 than *Triticum durum* and *vulgare*, but also, and this is the interesting point, than *Triticum monococcum*. With our method of P32 treatment the appropriate dose in the case of *vulgare* wheat should be less than 5 μC per seed but most probably above 1 μC/seed. *Triticum dicoccum* is even more susceptible. These concentrations, concluded out of laboratory experience, will be tested in future field experiments.

In conclusion, we want to stress once more that the results reported in this paper must be regarded as preliminary rather than definite. They imply a modest approach to the application of radioactive isotopes in plant-breeding and genetics from biochemical points of view.

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INDUCED BACK-MUTATIONS IN A SPECIFIC GENE OF NEUROSPORA CRASSA

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I. INTRODUCTION.

MULLER's discovery in 1927 of the mutagenic effect of X-rays on *Drosophila* marked the beginning of a new era in genetics. It started work on experimentally induced mutations, which was to be extended to other organisms. As a result, the mutagenic effect of radiation (X-rays, neutrons, ultra-violet light, *etc.*) was demonstrated beyond doubt. Nearly 20 years had to pass, however, before the mutagenic effect of a chemical agent could be demonstrated with the same certainty. The work of AUERBACH and ROBSON, first released after the war, showed, however, likewise first on *Drosophila*, the strong mutagenic effect of sulphur and nitrogen mustards; for references, see AUERBACH (1949). Their work was to be followed by an intensive search for other mutagenic chemicals, and a number of compounds are now on record which are claimed to have a more or less strongly mutagenic effect. The list of mutagenic compounds now includes phenol (see HADORN, ROSIN and BERTANI, 1949), phenol derivatives (LEVAN and TJIO, 1948), urethane (OEHLKERS, 1946; VOGT, 1948), a number of carcinogenic compounds like methylcholanthrene and others (DEMEREC, 1949), methylxanthines like coffeine and theophylline (FRIES and KIHLMAN, 1948), and several others (see also WITKIN, 1947).

We should like in this introduction to discuss two points which have a profound significance for the evaluation of the work on experimentally induced mutations. The first one deals with the proper definition of the mutation conception in cytogenetics.

The rapid development of the cytological technique, and especially the discovery of the polytene chromosomes in Diptera soon forced geneticists to adopt a broader mutation concept than was probably originally intended. We may at least for the present purpose define a mutation as a morphological, physiological or even a cytological alteration of the individual which is inherited, *e. g.* transmitted through the sexual cycle. It was soon discovered that such changes may arise through

many different mechanisms, and especially in *Drosophila* it was found that a great number of mutations were due to chromosome alterations, and not, as was originally believed, were caused by alterations or rearrangements within the gene molecule itself. Therefore the mutation concept covers, and has to cover, changes due to structural or numerical alterations of the chromosome set (the various types of chromosome mutations) as well as changes due to intragenic rearrangements (gene mutations). However, only in the most favourable organisms like *Drosophila* or *Zea* can it be decided whether a mutation is of the chromosomal type or whether it is a gene mutation. Even in such organisms we are forced to classify as gene mutations only such mutations as do not seem to be associated with visible chromosomal rearrangements when studied by the present cytological and microscopical technique (*cf.* STADLER, 1941).

This does not necessarily imply, as it has sometimes been claimed to do, that all mutations are due to chromosomal rearrangements or small, invisible deficiencies, and that true gene mutations caused by intramolecular changes simply do not exist. On the contrary, there is still good reason to believe that a great part of the spontaneous or induced recessive mutations which show normal Mendelian segregations are good gene mutations. On the other hand, the shortcomings of our technique in detecting and classifying specific gene mutations should not be underrated.

These considerations lead to the second problem which we want to raise here: On what basis are we to classify a physical or chemical agent as mutagenic?

Thanks to the development of the *Drosophila* ClB test, the work on experimentally induced mutations was from the very beginning put on a solid quantitative basis and the relationship between doses and mutation frequency revealed the mechanism by which mutations are induced by radiation.

The ClB test is in many respects ideal for the purpose. It is extremely sensitive; unbiased by any personal influence from the investigator, it scores the mutation rate of a single chromosome, not rearranged by crossing-over, *etc.* Even the difficulties of applying chemical agents to *Drosophila* sperms have largely been overcome, either by means of the transplantation technique used by HADORN and his coworkers, or by means of the aerosol technique developed by AUERBACH and by DEMEREC. No wonder, therefore, that by far the greatest part of the work on experimentally induced mutations has been based on this

test, and it is certainly no over-estimation to say *that a chemical or physical agent is classifiable as mutagenic if it is able to increase the number of lethals in the X-chromosome of Drosophila melanogaster when scored by the ClB test.*

As is well known, however, both radiation and the chemical mutagens induce a high number of chromosome breaks and chromosome rearrangements, and it has been shown by various authors that a high proportion of the lethals recorded by the ClB test are associated with, and probably due to, smaller or larger rearrangements or minute losses of a few chromosome segments (see CATCHESIDE, 1948, for summary). The effect of radiation or of the chemical mutagens on the chromosomes has been further studied on plant material, where the pollen tube mitoses of *Tradescantia* species (SAX, CATCHESIDE, DARLINGTON and KOLLER, and others; for references, see CATCHESIDE, 1948; LEA, 1946) or the root tip mitoses of *Allium* (LEVAN and co-workers; see LEVAN, 1949) have been the favoured material. *A number of chemical compounds have thus been described as mutagenic because they are able to induce chromosome mutations in these or other plants.*

However, the *Allium* and *Tradescantia* tests are exclusively tests for chromosome mutations, and the ClB method, although it is a more genetic test than the others, *is by no means a specific test for gene mutations*, far less it is possible by this method to study the mutability of individual genes.

In fact, although very much needed, quantitative data on the spontaneous and induced mutation rate of individual genes are extremely scarce. The most extensive data have been provided by STADLER and his co-workers, studying the spontaneous, X-ray and ultra-violet induced mutations of the »A» and the »R» loci of *Zea* (see STADLER and ROMAN, 1948; STADLER, 1946, *et al.*), and by the older data on the white locus of *Drosophila* (see TIMOFÉEFF-RESSOVSKY, 1937). This is of course due to the great stability of most wild type genes, which makes it necessary to investigate many thousands of individuals in order to obtain sufficiently accurate data on the mutation rate of a single locus.

Investigations into this problem on a larger scale will in fact only be possible under very specific experimental conditions, *viz.* with the use of a screening technique which automatically sorts out the non-mutated nuclei so that only the mutated cells are recorded. Such a screening technique has successfully been developed by LEWIS (1948) in studying the mutability of the »S» locus of self-sterile plants. The screening method also forms the basis of the work on phage and drug

resistance in bacteria (for references, see LEDERBERG, 1948). However, exceedingly important as the bacteriological work is from other points of view, it still falls short in the case of our problem in two respects: First, the genetical test, *e. g.* the Mendelian crossing experiment, which should not be omitted in mutation work, is still not sufficiently under control in bacteria, although the work of TATUM and LEDERBERG show that eventually it may become so (see LEDERBERG, *loc. cit.*). For the present, however, the possibility cannot be formally excluded that some of the »transformations» in bacteria, spontaneous or induced, may be modifications, »Dauermodifikationen», phenocopies, *etc.* Second, even if the transformations to drug or phage resistance are actually homologous to the mutations in higher organisms, we still do not know whether they arise through mutations of a single gene, or whether — and this seems more likely — they are due to mutations in several independently mutating genes.

It is the scope of the present paper to show that the mould *Neurospora*, which in recent years has yielded so many valuable contributions to genetics (see BEADLE and TATUM, 1941, *et al.*), offers favourable opportunities for studying the mutability, or more specificly the back-mutation rate of individual genes by means of a sieving technique similar to that used in bacteriological work. Such experiments with *Neurospora* would have the advantage of being carried out with an organism in which the genetical crossing experiment is sufficiently under control (DODGE, 1927). The technique to be described records the back-mutation rate of the so-called biochemical mutants of *Neurospora*, *e. g.* mutant strains in which the capacity to carry out a specific enzymatic reaction necessary for the normal growth of wild type strains is blocked by mutation (BEADLE and TATUM, 1941). Hence the biochemically deficient strain will grow only if the specific substance normally produced by the biochemical reaction is added to the culture medium.

It has been found that some of the strains carrying biochemical mutants will sometimes, sooner or later, revert to wild type again, *e. g.* they will again grow on the »minimal medium» (see BEADLE and TATUM, 1945), not containing the specific substance in question. This reversion to wild type growth has been found to depend upon different mechanisms in different strains (see RYAN, 1946; GILES and LEDERBERG, 1948). In some cases the reversion is due to a non-genetic adaptation, but in other strains it can be proved genetically that the reversion is due to a back-mutation of the biochemical mutant to wild type. This back-

mutation rate is easily scored by means of a screening technique: Conidia from a biochemically deficient strain are plated on minimal medium. No growth takes place except from spores in which a back-mutation to wild type has taken place; hence only from such spores will visible colonies be formed.

The experiments were started in 1946—47 by the senior author (WESTERGAARD) when working at the Kerckhoff Laboratories of the California Institute of Technology as a Rockefeller Research fellow, and he takes the opportunity to express his sincerest thanks to Dr. G. W. BEADLE and to the staff of the Department for placing the cultures at his disposition and for encouraging interest in the work. Special thanks are due to Dr. H. MITCHELL and to Mrs. MARY HOULAHAN for their great help and many valuable suggestions for these experiments. The experiments were interrupted for almost a year after the senior author's return to Denmark, but were then continued in collaboration with KÖLMARK. They have been supported by grants from the Rockefeller Foundations and from the Carlsberg Foundations.

In the meantime there appeared the important paper by GILES and LEDERBERG (1948) in which they study the back-mutation rate of several biochemical *Neurospora* mutants by a technique somewhat similar to that used by us. We have therefore decided to omit most of our data on the effect of X-rays and ultra-violet light on the mutation rate of the strain investigated here, since the results largely coincide with the data of the American authors, and in the present publication we give only the data on the effect of nitrogen mustard. Experiments with other chemical compounds will be published later (JENSEN, KÖLMARK and WESTERGAARD, 1949).

II. MATERIAL AND METHODS.

The biochemical mutant used in these experiments is an adenine-requiring strain, obtained originally from ultra-violet treatment (key number 38701 adenineless; see MITCHELL and HOULAHAN, 1946). The strain was known to revert to wild type occasionally. The adenineless strain has the spreading growth characteristic of wild type *Neurospora*; and when plated out, it will cover the whole petri dish in a very short time. For this reason it cannot be plated for the counting of colonies by the standard microbiological plating technique. Therefore the adenineless strain was crossed to a morphological mutant (key number 70007 A, originally obtained from mustard gas treatment) which had an altered growth habit, the mycelium forming small dense colonies, which can easily be counted by the usual plating technique. From this cross the double mutant, adenineless, colonial growth, was recovered. This strain, W. 40, mating type *a*, has been used in the experiments, it forms small distinct colonies on the agar surface when plated on a petri dish, and even after 4—5 days the colonies will not overgrow each other. No

reversion of the colonial mutant to wild type spreading growth has been found.

The general procedure of the experiment is the following: 30—40 test-tubes with »complete medium», *e. g.* medium containing adenine (see BEADLE and TATUM, 1945) are inoculated with the W. 40 strain and incubated for 4—8 days at 25° C. The spores, *e. g.* the asexual, multinucleate macroconidia are then suspended in sterile water, and afterwards filtered through absorbing cotton, which holds back small pieces of mycelium. The filtration is done in a Büchner funnel under application of suction. From the homogeneous spore suspension 8 cc are pipetted into a number of sterile centrifuge tubes, and the spores are centrifuged for two minutes at 3500 revolutions. The water is then replaced by 8 cc of the nitrogen mustard solution and treated under the conditions required by the experiment. The nitrogen mustard crystals are dissolved in a sterile buffer. A commercial sample of nitrogen mustard (methyl-bis-β-chloroethyl-amine-hydrochloride) »Erasol» obtained through the courtesy of »Ferrosan», Inc. of Copenhagen was used. The suspension is put on a shaker machine and agitated for the time required by the experiment. The spores are then centrifuged again, and the reaction is stopped by replacing the nitrogen mustard by 8 cc of a glycine-bicarbonate solution (60 mg. glycine+68 mg. of sodium bicarbonate to 100 cc destilled water; see STAKMAN and STAUFFER, 1947), to be followed by two washings in sterile water. Finally, 6 cc of sterile water are added to the spore precipitate.

Out of the 6 cc of spore suspension, 1 cc is diluted in sterile water (normally from 1×10^4 up to 1×10^6, depending upon the treatment) and from a suitable dilution 5 cc of the diluted suspension are plated on ten petri dishes with »complete medium», 0,5 cc being added to each plate, and spread by means of a glass needle. The remaining 5 cc of the concentrated spore suspension are plated on ten petri dishes containing minimal medium.

On the ten »complete» dishes, inoculated from the diluted spore suspension, all spores will grow and form colonies which have survived the treatment, because the complete medium contains the adenine which is necessary for the growth of the mutant. Hence on the ten »complete» dishes can be counted: (1) The number of spores initially present in the control experiment, and (2) The numbers of survivers in the treated series. On the ten »minimal» petri dishes inoculated from the non-diluted spore suspension only wild type spores will form colonies, since the minimal medium does not contain adenine. Hence

only those spores will grow to form visible colonies in which a back-mutation of the adenineless mutant gene to the adenine synthesizing wild type gene has taken place. From these two sets of experiments can thus be calculated: (1) The number of spores originally present; (2) The number of spores which have survived the treatment; (3) The back-mutation rate per initial number of spores; and (4) The back-mutation rate per surviving spores.

Finally, a number of the colonies formed on the minimal medium have been isolated and crossed to wild type of the opposite mating type. The results will be described in a later paragraph.

III. EXPERIMENTAL RESULTS.

For the evaluation of the following tables two factors should be emphazised. First: The mutation rate scored in the untreated control experiments are the number of back-mutations which have taken place between the time of inoculation of the test-tubes and until the spores are harvested for the experiment, whereas the mutations recorded in the treated series are the number of back-mutations induced by the treatment plus the spontaneous mutations. *The spontaneous back-mutation rate of the adenine locus is thus not known, and cannot easily be defined.* Second: All the experiments have been done with multinucleate macroconidia, with the number of nuclei probably varying from 1—12, and with 3—4 nuclei per conidium being about the average. *Hence the figures in the tables do not give the back-mutation rate of the gene, but only the mutation rate per number of spores.* The figures should thus be divided by a factor of 3—4 to give the true back-mutation rate of the gene.

(a) *Influence of the hydrogen ion concentration on mutation yield.* — A careful control of the hydrogen ion concentration is indispensable in all experiments with nitrogen mustard, since its reactivity increases when the solution becomes more alkaline (see GILMAN and PHILIPS, 1946, for references). By means of the »Universalpuffer» of TEORELL and STENHAGEN (1938), which covers a pH range from 2,0 to 12,0, this factor can be controlled with sufficient accuracy. The pH of the buffer is checked before and after autoclaving, the values given in Table 1 correspond to the pH values after autoclaving. As appears from the table, the yield of mutations is determined by a balance between the killing effect of the nitrogen mustard and the mutagenic effect, both increasing as the solution becomes more alkaline.

TABLE 1. *pH experiment, temperature 26° C., 30 minutes' treatment, 1/200 mol. nitrogen mustard, yield scored after 4 days.*

Treatment	Numb. of viable spores	% survived	Numb. of mutations	Mutations per 10^6 initial spores	Mutations per 10^6 survived
Untreated (control)	$47{,}0 \times 10^6$	100	1	0,021	0,021
pH 2,0	37×10^6	79	4	0,085	0,11
» 4,2	$27{,}5 \times 10^6$	59	5	0,11	0,18
» 6,1	$7{,}76 \times 10^6$	17	8	0,17	1,0
» 8,0	$1{,}16 \times 10^6$	2,5	8	0,17	6,9
» 9,9	$0{,}71 \times 10^6$	0,15	14	0,30	19,7
» 11,6	$0{,}09 \times 10^6$	0,019	5	0,11	55,5

(b) *The effect of nitrogen mustard concentration on the yield of mutations.* — The figures are grouped in Table 2. Concentrations stronger than 1/100 mol. are completely lethal to the spores. The highest yield of mutations is actually obtained from treatments with rather low concentrations, and even 1/1600 mol. nitrogen mustard has a pronounced mutagenic effect. This is of course again explained by the high toxicity of the strong concentrations, by which some of the back-mutated spores are also killed by the treatment.

TABLE 2. *Concentration experiment, temperature 25° C., 30 minutes' treatment, pH 9,5, yield scored after 4 days.*

Treatment	Numb. of viable spores	% survived	Numb. of mutations	Mutations per 10^6 initial spores	Mutations per 10^6 survived
Untreated (control)	$73{,}00 \times 10^6$	100	2	0,027	0,027
1/100 mol. nitrogen mustard	$0{,}0025 \times 10^6$	0,0034	1	0,014	400,0
1/200 mol. nitrogen mustard	$0{,}58 \times 10^6$	0,79	13	0,18	22,4
1/300 mol. nitrogen mustard	$2{,}30 \times 10^6$	3,2	17	0,23	7,4
1/400 mol. nitrogen mustard	$4{,}20 \times 10^6$	5,8	19	0,26	4,5
1/600 mol. nitrogen mustard	$14{,}00 \times 10^6$	19	22	0,30	1,6
1/800 mol. nitrogen mustard	$21{,}80 \times 10^6$	30	24	0,33	1,1
1/1200 mol. nitrogen mustard	$55{,}00 \times 10^6$	75	19	0,26	0,35
1/1600 mol. nitrogen mustard	$71{,}50 \times 10^6$	98	14	0,19	0,20

TABLE 3. *Influence of duration of treatment, temperature 20° C., 1/200 mol. nitrogen mustard, pH 9,5, yield scored after 4 days.*

Time treated	Numb. of viable spores	% survived	Numb. of mutations	Mutations per 10^6 initial spores	Mutations per 10^6 survived
Untreated (control)	68 $\times 10^6$	100	0	0	0
5 minutes	27,8 $\times 10^6$	41	12	0,18	0,43
10 »	20,2 $\times 10^6$	30	15	0,22	0,74
20 »	19,7 $\times 10^6$	29	26	0,38	1,32
40 » . ..	7,40 $\times 10^6$	11	30	0,44	4,01
80 »	4,84 $\times 10^6$	7	24	0,35	4,96
160 »	1,98 $\times 10^6$	3	21	0,31	10,61
320 »	0,64 $\times 10^6$	0,9	16	0,24	25,00

(c) *The effect of duration of treatment upon mutation yield.* — These experiments were carried out at a somewhat lower temperature (20° C) than the other experiments. Again, the relative yield of mutations increases proportionally to the time of treatment (Table 3).

TABLE 4. *Temperature experiment, 30 minutes' treatment, 1/200 mol. nitrogen mustard, pH 9,5, yield scored after 4 days.*

Treatment	Numb. of viable spores	% survived	Numb. of mutations	Mutations per 10^6 initial spores	Mutations per 10^6 survived
Untreated (control)	100,0 $\times 10^6$	100	2	0,020	0,020
4° C.	4,0 $\times 10^6$	4,0	16	0,16	4,00
15° »	3,7 $\times 10^6$	3,7	22	0,22	5,95
25° »	0,24 $\times 10^6$	0,24	20	0,20	83,30
35° »	0,08 $\times 10^6$	0,08	13	0,13	162,50

(d) *The effect of temperature upon mutation yield.* — Temperatures above 35° C are lethal to the spores, but within the range of temperature tolerance, the yield of back-mutations is very much influenced by temperature (Table 4).

(e) *Inactivation of the nitrogen mustard by the buffer.* — Since nitrogen mustard already reacts with water, it is of interest to see how fast the mutagenic activity is lost after the reaction is started. The experiments are shown in Table 5. A 1/200 mol. nitrogen mustard solution at pH 9,5 was prepared and allowed to react at 25° C. Eight cc of this solution were added to a series of spore precipitates at intervals of 5, 10, 20, *etc.* minutes, and allowed to react with the spores for 30 minutes. The results are somewhat surprising. For one thing, the nitrogen mustard is still mutagenic after having reacted with the buffer for 80

TABLE 5. *Inactivation experiment, temperature 26° C., 30 minutes' treatment, 1/200 mol. nitrogen mustard, pH 9,5, yield scored after 4 days.*

Nitrogen mustard allowed to react in buffer for	Numb. of viable spores	% survived	Numb. of mutations	Mutations per 10^6 initial spores	Mutations per 10^6 survived
Untreated (control)	$47{,}0 \times 10^6$	100	1	0,021	0,021
0 minutes	$0{,}71 \times 10^6$	0,15	14	0,30	19,70
5 »	$4{,}02 \times 10^6$	9	31	0,66	7,7
10 »	$7{,}3 \times 10^6$	16	32	0,68	4,4
20 »	$18{,}7 \times 10^6$	40	26	0,55	1,4
40 »	$26{,}0 \times 10^6$	55	25	0,53	0,96
80 »	$43{,}5 \times 10^6$	93	9	0,19	0,21

minutes. Next, the yield of mutations per million initial spores is higher in this experiment than in any other. Whether this suggests that the toxic effect of nitrogen mustard is inactivated faster by the buffer than is the mutagenic effect, or whether it is due to mere chance, still has to be investigated.

In summarizing these experiments it can be concluded that the yield of back-mutations in the experiments depends upon the following factors: (1) The initial hydrogen ion concentration in the experiment; (2) Nitrogen mustard concentration; (3) The time during which the nitrogen mustard is in contact with the spores; (4) Temperature; (5) The age of the nitrogen mustard solution.

This is of course what would be expected from the chemical properties of nitrogen mustard (see GILMAN and PHILIPS, 1946) and from the behaviour of chemical reactions in general. What should be pointed out here, however, is that all these factors can be sufficiently controlled by the experimental technique here employed, since within each set of experiments a clearcut correlation is found between the yield of back-mutations

TABLE 6. *Yield of mutations in different experiments with 1/200 mol. nitrogen mustard under standard conditions (pH 9,5, 30 minutes' treatment, 25° C.).*

% survived	Mutations per 10^6 initial spores	Mutations per 10^6 survived
0,15	0,30	19,7
0,24	0,20	83,3
0,79	0,18	22,4
14,5	0,54	3,8
4,7	0,22	4,7
1,6	0,11	6,7
3,04	0,26	6,9

and the variable factor of the experiment. When, on the other hand, different sets of experiments are compared, it is evident that it is very difficult to reproduce the exact yield from one experiment to another. Table 6 gives the yield obtained from 1/200 molar nitrogen mustard treatment under standard conditions in different experiments. Part of the variation encountered in this table must undoubtedly be ascribed to experimental errors, since the yield is very sensitive to even small changes in temperature or pH, both of which factors it is difficult to reproduce quite exactly with our present facilities.

We believe, however, that most of the variation is due to other factors. Among these undoubtedly the age and condition of the conidia when harvested for the experiment play a dominant part, as already shown by MILLER and MCELROY (1948). We believe that especially the killing rate of the spores is very much influenced by these factors, which have not been sufficiently controlled, and the experiments should undoubtedly be made with 3—4 days old conidia taken from cultures which have been grown under careful control of temperature and humidity.

Another factor which influences the yield of mutations is the nitrogen mustard sample. The best yield is obtained from freshly prepared, specially purified crystals, but we have not been able to use such samples for all the experiments.

With these sources of errors kept in mind, the back-mutation test seems to be sufficiently sensitive and accurate to serve as a useful tool for investigating the mutagenic activity of water-soluble compounds as well as for studying the mutation rate of specific genes.

IV. CROSSING EXPERIMENTS.

Table 7 gives the results of the crosses, in which a number of reverted colonies from the minimal medium has been crossed to wild type strains of the opposite mating type, using the special crossing medium developed by WESTERGAARD and MITCHELL (1948). From the perithecia produced from these crossings the spores were dissected from the asci in linear order, using the dissection technique described by BEADLE and TATUM (1945). One ascus only was dissected from each perithecium. The spores were germinated on complete medium and afterwards growth was investigated on the minimal medium. The spores which did not grow on the minimal medium were classified as adenine-less, those which grew were classified as wild type.

TABE 7.

Culture number	From treatment with	Number of asci dissected	Asci with 8 wild type spores	Asci with 4 wild type spores and 4 adenineless spores
1	ultra-violet light	4	4	0
2		3	0	3
3		3	3	0
4		6	0	6
5		3	3	0
6		3	1	2
7		1	1	0
8		8	6	2
9		4	3	1
10		2	0	2
11		6	6	0
12		5	2	3
13	X-rays	5	5	0
14		2	0	2
15		1	0	1
16		4	1	3
17		3	0	3
18		6	0	6
19		2	0	2
20		2	0	2
21		10	8	2
22		10	10	0
23		5	1	4
24		5	5	0
25		5	3	2
26	nitrogen mustard	4	3	1
27		3	3	0
28		6	5	1
29		5	3	2
30		2	2	0
31		3	3	0
32		5	1	4
33		5	4	1
34	spontaneous	5	5	0
35		3	2	1

A priori it is to be expected that, if the reversion to wild type is due to a back-mutation, the reverted colonies would be heterocaryons containing both wild type and adenineless nuclei (see BEADLE and COONRADT, 1944). The heterocaryon would arise in two ways. The

macroconidia are usually multinucleate, and most likely only one of the nuclei in the spores will undergo a back-mutation. This will therefore form a heterocaryon with the other non-mutated adenineless nuclei in the spore. Even if the colony arose from a back-mutated uninucleate spore, a heterocaryon would be formed on the agar plate from fusions between the growing mycelium and the many non-mutated spores in the densely inoculated plate, because even the non-mutated spores of the biochemically deficient *Neurospora* strains send out small germinating tubes. The results of the crosses are in accordance with the heterocaryon hypothesis. Twelve colonies (1—3—5—7—11—13—22—24—27—30—31—34) have only given asci with 8 wild type spores, indicating that only the back-mutated spores in the heterocaryon have been recovered from the crossing. In 14 cases (6—8—9—12—16—21—23—25—26—28—29—32—33—35) both wild type and adenineless spores were recovered, since some asci gave 8 : 0 segregations whereas other asci from the same cross gave 4 : 4 segregations. Finally, 9 crosses gave only 4 : 4 segregations, indicating that only the adenineless nuclei had been recovered. The second possibility that the reversion in the latter group had been due to non-genetic factors does not seem very likely, and the most probable conclusion from the crossing experiments is that the reversion to wild type growth of the adenineless strain on minimal medium is due to a back-mutation in the adenine locus, the back-mutated wild type nucleus forming a heterocaryon with the non-mutated nuclei.

However, one other possible explanation of the reversion phenomenon should also be pointed out, *viz.* that it is due to an epistatic suppressor gene, which in some way or other suppresses the effect of the adenineless gene and enables the strain to carry out the adenine synthesis in a different way (compare HOULAHAN and MITCHELL, 1947). If, however, the suppressor gene (*S*) was carried on a different chromosome from the adenineless gene (*ad*), the adenineless character would still be recovered from the crossing experiments in the ratio 3 : 1 [*S,ad*×*s,Ad* would give 1 *S,Ad* (wild type) : 1 *s,Ad* (wild type) : 1 *S,ad* (wild type) : 1 *s,ad* (adenineless)]. Such 3 : 1 segregations have not been found in the crossing experiments. If, on the other hand, the hypothetic suppressor gene was closely linked to the adenineless gene, segregations of adenineless without the suppressor gene by crossing-over would be very rare, and the explanation of the reversion being due to a closely linked suppressor gene can at the present time not be at least theoretically rejected.

V. DISCUSSION.

(a) *Comments on the technique.* — The technique here described may be modified in one important respect by using *Neurospora* strains which only produce uninucleate microconidia. A number of such mutants are available (see BARRATT, GARNJOBST and TATUM, 1949). In such strains the mutation rate per number of spores would correspond directly to the mutation rate of the gene. However, microconidia of *Neurospora* are in other respects less suitable for studying the effect of very toxic compounds because they are killed off much faster than the multinucleate macroconidia. Besides, if part of the killing mechanism of mutagenic chemicals depends upon a nuclear killing by induction of lethals, all the nuclei of the spores would have to be killed before the spore is killed, whereas it is sufficient that only one of the nuclei back-mutates to start the spore growing. Therefore by using macroconidia we should have a more sensitive test especially for studying the effect of highly toxic compounds. On the other hand, it should be pointed out that a comparison of the mutation rate of the same gene from uninucleate and from multinucleate spores would be very important.

Finally it should be pointed out that the recent discovery that some surface-active compounds induce colonial growth of wild type *Neurospora* by modification (Miss GARNJOBST, personal communication) makes it possible to apply the plating technique to the biochemical strains directly without having to go through the trouble of first making the cross to a colonial growing strain.

(b) *Theoretical comments.* — The reversion to wild type growth of the adenineless strain is, as already stated, most likely explained by the hypothesis of a back-mutation of the recessive adenineless gene to the dominant wild type gene (*ad* → *Ad*). Since this is a very important point for the theoretical implications of the experiments, it should be pointed out once more that two other explanations are still possible. One, *viz.* the induction of a suppressor gene closely linked to the adenineless gene, can, as stated above, at the present time not be experimentally rejected. The other possibility is that the adenineless effect is a position effect, caused by, for instance, a small inversion. The reversion to wild type growth would then be explained by a reversion to the normal gene sequence of the chromosome. Position effects have not been discovered so far in *Neurospora*, and no visible cytological irregularities have been observed in the adenineless strain in question, but on the other hand it would be very difficult to detect, for instance, short inversions in *Neuro-*

spora cytologically or genetically. Although theoretically possible, both explanations seem far less probable than the back-mutation hypothesis.

If we accept the back-mutation hypothesis, the experiments have some theoretical implications. For one thing, the original adenineless mutation, which was induced by ultra-violet light, must have been due to an intramolecular rearrangement of the gene molecule, and not due to a mere loss of a minute piece of the chromosome, since it would be difficult to understand how a mutation of the latter type could be reversible. This implies again that, since both X-rays, ultra-violet light and mustard gas can increase the back-mutation rate, the effect of these three agents on the chromosomes cannot only be that of inducing chromosome breaks and chromatin deficiencies; they must also be able to induce reversible intramolecular changes. This is especially interesting in the case of X-rays, since it has been suggested, especially by STADLER (1941 *et seq.*), that X-rays, in contradistinction to ultra-violet light, has a mere destructive effect on the gene material. This hypothesis can hardly be maintained in the light of the present experiments. It would, on the other hand, be very interesting to investigate whether biochemical mutants derived from X-ray treatment back-mutate less readily than those derived from ultra-violet treatment or from mustard gas or *vice versa*. It is known that a great number of the biochemical mutants do not back-mutate spontaneously, and at least one strain has been studied (GILES and LEDERBERG, 1948) in which reversion to wild type could not be induced, and of course the above considerations do not imply that all biochemical mutations are gene mutations, and for that reason it does not seem likely, either, that all intramolecular rearrangements should necessarily be reversible. However, for a better understanding of these problems, it would be of great value to obtain statistical data on the mutation frequency from the dominant wild type to the recessive condition in which the biochemical synthesis of a specific reaction is blocked. However, it would appear to be a very laborious task to obtain information on this point in the case of the *Neurospora* mutants.

In conclusion we want to emphasize that the back-mutation test may be regarded as a more specific test for gene mutations than the ClB test and the *Allium* test. It would therefore not be surprising if chemical compounds that have a mutagenic effect when studied by the ClB method may fail to give any results when studied by the back-mutation test, and in fact such compounds have already been found (see JENSEN, KÖLMARK and WESTERGAARD, 1949). For quantitative studies of the mutagenic effect of radiation and of water-soluble chemicals, in some

cases also of ether-soluble compounds (see JENSEN, KÖLMARK and WESTERGAARD, 1949), the test may turn out to be a useful supplement to the ClB method and other tests for mutagenic activity.

SUMMARY.

A method has been described for scoring the back-mutation rate of a specific gene, adenineless, in *Neurospora crassa* by means of a screening technique.

The back-mutation rate of the gene can be increased by X-rays, ultra-violet light and by nitrogen mustard treatment.

The yield of back-mutations in the nitrogen mustard experiments depends upon the pH, temperature, concentration, duration of treatment, age of the solution, age and condition of the spores, and the purity of the nitrogen mustard sample.

The results are discussed in relation to the theory of spontaneous and induced gene mutations, and it is emphasized that the back-mutation test is a more specific test for gene mutations than the ClB test, the *Allium* test or other methods for determining the mutation rate.

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NOTES ON THE APPEARANCE OF NEW BIOTYPES CLOSELY RELATED TO MALVA PARVIFLORA L.

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IN a culture of *Malva parviflora* in the Botanical Gardens of Upsala, there appeared in the year 1896 a plant that in the form of its leaves occupied an intermediary position between *M. parviflora* and the *M. oxyloba* growing close by. During the following summer there grew out of its seeds, besides *M. parviflora*, a great number of plants that in leaf-structure occupied an intermediary position between *M. parviflora* and *M. oxyloba*. They did not resemble each other completely. Some of the plants occupied a clearly intermediary position between them, and fruits were collected from one of these, to be sown another year. From the seeds plants then grew that were altogether similar to each other as to the form of their leaves, thus constituting a definite biotype. In the years 1902 and 1903 seeds from this biotype as well were distributed in the seed-catalogue of the Botanical Gardens of Upsala under the name of *M. parviflora-oxyloba*. This biotype is hereafter referred to under the name of *M. intermedia*, and it is reproduced in the Fig. 1 with the leaf *b* between the leaves *a* of *M. parviflora* and *c* of *M. oxyloba*.

The new biotype that appeared in 1896 in a culture of *M. parviflora* and that has been given the name *M. intermedia* could not have come into existence independently of *M. oxyloba*, as in multiplications of *M. parviflora* hybrids with a new biotype never appeared when the seeds for its multiplication had been collected from plants that were protected against insects by means of tarlatan and the seed-formation of which could thus only take place by its own pollen. The cause of the appearance of *M. intermedia* evidently was that pollen from *M. oxyloba* had been transferred to *M. parviflora* by insects and that the gene determining the laciniation of the leaves had undergone a mutation in the male sex-cell united with a female sex-cell in *M. parviflora*, thus producing the leaf-structure characteristic of *M. intermedia*. A leaf of this new biotype is depicted in Fig. 1 at *b*. A leaf of *M. parviflora* is

shown at *a*, whereas *c* represents a leaf of *M. oxyloba*. The new biotype occupies such an intermediary position between these two that it would be supposed to be a hybrid between them if only the form of the leaves were considered.

Several new biotypes appeared in the subsequent propagation of the three biotypes already mentioned. In the majority of the cases they owed their origin to mutations in the gene in their sex-cells determining the laciniation of the leaves, making the leaves either more weakly or more strongly laciniated in the biotype originated by such a mutation. As to the laciniation of the leaves, the biotypes thus originated, together with the two original biotypes, constituted a series of forms possessing characteristics that may be discerned by comparing with each other the 6 leaves depicted in Fig. 1 and presenting the characteristics of each of 6 different biotypes. Each of these leaves is the eighth or ninth leaf of plants that have grown under the same external conditions and are thus genotypically comparable with each other.

The names I have given the 6 biotypes, the leaves of which are designated *a*—*f* in Fig. 1, are the following: *a*, *M. parviflora*; *b*, *M. intermedia*; *c*, *M. oxyloba*; *d*, *M. digitata*; *e*, *M. tenuifolia*; *f*, *M. rediviva*. Out of these leaves, *a*—*e* constitute a series of biotypes with successively more strongly laciniated leaves. Another two biotypes were obtained in the cultures. In leaf-structure one of them occupied a position between *c* and *d*, the other between *d* and *e*. As was mentioned above, the mutations to which these biotypes owed their origin caused the appearance of either more weakly or more strongly laciniated leaves. The leaf *f* presents an instance of a mutation into more weakly laciniated leaves than those characteristic of *M. parviflora*. In the series of differently laciniated leaves the place of *f* is hence to the left of *a*. It is remarkable that mutations can take place in such a way as to produce new biotypes that, together with those already existing, constitute a series of biotypes characterized by gradually varying laciniation of the leaves.

Some of the numerous cultures continued up to the year 1938 will be mentioned in the following in order to explain the origin of the new biotypes shown in Fig. 1. The biotypes are designated with the letters given to their leaves in Fig. 1. For instance, a hybrid between *M. intermedia* and *M. oxyloba* is designated $b \times c$ and its offspring $b + c + + b \times c$. These letters may also designate the genes. In this case the hybrid mentioned would have been designated *bc* and its offspring $bb + cc + bc$.

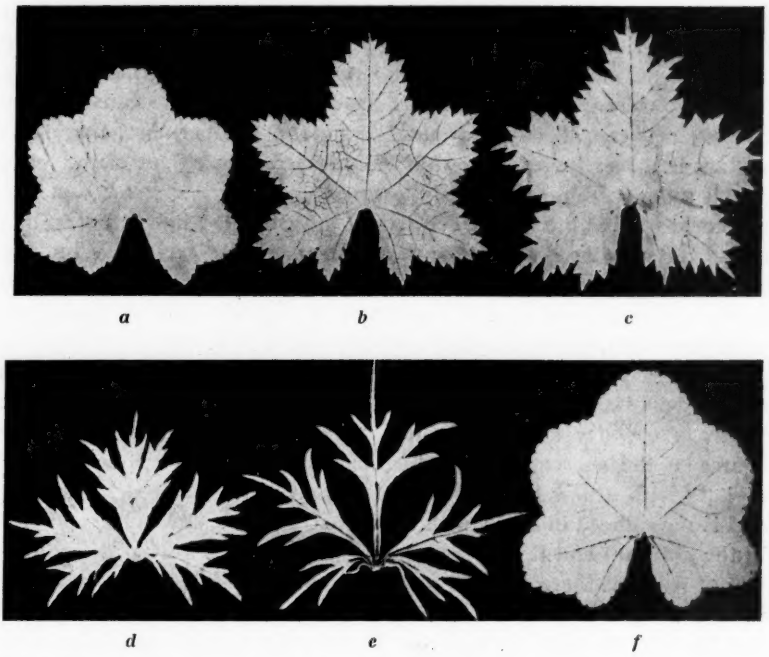


Fig. 1. The eighth or ninth leaf of the main shoot of 6 biotypes: *a*, *M. parviflora*; *b*, *M. intermedia*; *c*, *M. oxyloba*; *d*, *M. digitata*; *e*, *M. tenuifolia*; *f*, *M. rediviva*. They all grew under the same external conditions.

The appearance of *M. intermedia* gave the impulse to further cultivation of the three biotypes reproduced at the top of Fig. 1. These investigations were carried on till the year 1938. On several occasions other types appeared besides *M. intermedia*, the three most common such being reproduced in the lower row of Fig. 1, *viz. M. digitata* with the leaf *d*, *M. tenuifolia* with the leaf *e* and *M. rediviva* with the leaf *f*. The leaves depicted were the eighth or ninth leaf of the main shoot of plants that had grown under similar external conditions.

In the course of these investigations the first observation of the appearance of a new biotype was made in 1909. In a culture of *M. oxyloba* there was a plant with deeper laciniation of the leaves than that of the other plants. Evidently a new biotype was at hand in the form of a hybrid with *M. oxyloba*. Out of its seeds grew 69 plants. Of these, 17 had leaves of the form given as *d* in Fig. 1, and continued multi-

plication showed them to constitute a new biotype, which was given the name *M. digitata.* Out of the 69 plants, 15 had the leaf-structure *c* and consisted of *M. oxyloba.* Of the 69 plants constituting the offspring of the hybrid $c \times d$, 36 resembled the mother and were consequently hybrids between *M. oxyloba* and *M. digitata.* Furthermore, there was 1 plant that had leaves somewhat less divided than *c.* Out of the seeds of this plant there grew in the following year $2\ b + 3\ c + 2\ b \times c$. The gene determining the leaf-structure had thus mutated into *b*, but in this instance it was probably not *c* that had undergone this mutation, but in all probability *d* had mutated into *b*. As a reason for this assumption it may be mentioned that in a cross of *M. digitata* with *M. parviflora* there appeared, besides hybrids $a \times d$, one $a \times b$.

After a subsequent sowing, in the summer of 1910, of the seeds collected in 1909 from the plant $c \times d$, there also appeared numerous plants of $c + d + c \times d$, but there appeared a plant, too, that in the course of the subsequent investigations proved to be a hybrid of *M. digitata* with a new biotype, which was given the name *M. tenuifolia* and is reproduced in Fig. 1 with the leaf-structure *e*. It was transplanted in the autumn, and it flowered and fruited indoors during the winter. Out of its seeds there grew 32 plants in the summer of 1911, 8 of which were *M. digitata.* Twenty plants evidently consisted of *M. tenuifolia* and its hybrids with *M. digitata.* There were 4 other hybrids, which could not be determined solely from their appearance. When *M. tenuifolia* first appeared out of the heavily laciniated *M. digitata*, it was not possible to distinguish it from its hybrids with the latter. This was, however, easily done when it originated from a biotype characterized by less divided leaves. This was the case in the following series of experiments.

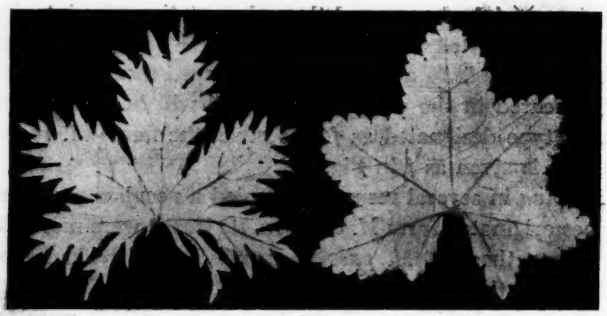
After a sowing of seeds obtained from some plants in a culture of *M. oxyloba* in 1929, numerous plants grew up which, if designated with the alphabetic signs for their leaves given in Fig. 1, consisted of $113\ c + 3\ c \times b + 1\ c \times e$. In 1932, the hybrid $c \times e$ thus obtained produced $5\ c + 6\ e + 14\ c \times e$. The offspring of 1 *e* in this culture was in 1933: $4\ e + 2\ e \times b + 1\ e \times c$. In 1934, this hybrid $e \times c$ gave rise to the following plants: $4\ e + 7\ c + 8\ e \times c$. One out of these 4 *e* was completely sterile. Two others had, besides leaves of the form *e*, a few leaves resembling *f* (in Fig. 1). Fruits were collected from the parts of the stalks of one of these two plants where only leaves of the normal form *e* were present. The following plants grew out of the seeds of these fruits in 1936: $9\ e + 1\ e \times f + 1\ e \times c + 2\ e \times ?$. Here a new

biotype, *viz.* $e \times f$, had appeared through mutation of *e* into *f*. Its offspring in 1937 consisted of 48 plants, *viz.* 11 *f* + 13 *e* with changed leaf-structure + 24 $e \times f$. An account of the changed forms of the leaves in which *e* appeared in the offspring of $e \times f$ will be given further on. The new biotype designated as *f* was given the name *M. rediviva*. Its leaf-structure is given as *f* in Fig. 1.

M. rediviva in several respects occupied a peculiar position among the biotypes originated from *M. oxyloba*. Its leaves were more shallowly laciniated than those of *M. parviflora*, which had evidently been the most original of the biotypes mentioned. *M. rediviva* differed from the latter by its leaves having much shorter laciniae, which were to a high degree roundedly obtuse. (In *M. parviflora* they could be somewhat more obtuse than in Fig. 1 *a* when the plant had become more strongly developed.) *M. rediviva* was already recognizable at the germinal shoot stage by the lowest leaves of the main shoot lacking deep indentations and being but shallowly crenate. Further differences between these two biotypes will be pointed out in the account of the vegetative mutations that may appear in *M. tenuifolia*.

In the numerous cultures of the biotypes originating from *M. oxyloba*, only *M. tenuifolia* could produce *M. rediviva*. It is remarkable that it was also the only biotype that could reproduce *M. parviflora* by mutation. From its offspring referred to above, containing 9 *e* besides 1 $e \times f$, fruits were collected from a plant *e* for propagation later on (1937). This plant as well as 3 other *e* and the hybrid $e \times f$ had come out of seeds from one and the same fruit of the mother-plant *e*. Its offspring consisted of 13 *e* + 1 $e \times a$ + 5 $e \times f$. In the following year, $e \times a$ produced 10 *a* + 5 *e* + 20 $a \times e$, and 1 $e \times f$ gave rise to 7 *f* + 1 *e* + 11 *e* with changed leaf-structure + 18 $f \times e$. The plants *e* originating from $e \times a$ presented the leaf-structure characteristic of *e*, but the plants *e* originating from $e \times f$ with one exception presented a changed leaf-structure, as was generally the case in multiplication of this hybrid. It may be mentioned that the appearance of the hybrids $e \times a$ and $e \times f$ could not be due to crossing with free-growing *a* and *f*, as the plants from which fruits were to be collected for further cultivation and investigations were always kept guarded against insect visits by means of tarlatan from the time of their flowering. The appearance of hybrids in the offspring of a non-hybrid plant was thus always caused by mutation of a gene.

A considerable difference between *M. parviflora* (*a*) and *M. rediviva*



g *h*

Fig. 2. The ninth leaf of the main shoot of two hybrids: *g* of *M. tenuifolia* × *parviflora*, and *h* of *M. tenuifolia* × *rediviva*.

(*f*) was observed in their hybrids with *M. tenuifolia* (*e*) in the cultivation of the latter.

The leaf-structure of the hybrid *a* × *e* is reproduced in Fig. 2 with the leaf *g*. The influence exercised by *e* has been strong in the formation of the structure of this leaf. In the formation of the leaf-structure of the hybrid *e* × *f*, here reproduced with the leaf *h*, however, the biotype *f* has exercised a much stronger influence than *e*. As previously mentioned, the biotypes depicted in Fig. 1 are arranged from *a* to *f* in such a way that in a case of crossing between two of them the biotype situated to the right has a stronger influence on the leaf-structure of the hybrid than the one situated to the left.

Yet another difference is to be noticed between *M. parviflora* (*a*) and *M. rediviva* (*f*) in their hybrids with *M. tenuifolia* (*e*). In the offspring of *e* × *a*, *e* had the leaf-structure determined by the gene *e*, but in the offspring of *e* × *f* the leaf-structure of *e* was determined by a factor made active in this biotype by *f*. This change of form in *M. tenuifolia* will be dealt with later.

The tendency of *M. tenuifolia* to produce other biotypes by means of mutation seems to be intensified in different degrees by crossing with certain other biotypes. Numerous investigations, a small part of which have been previously mentioned, seem to indicate that the crossing of *M. tenuifolia* with *M. oxyloba* and *M. digitata* increases this tendency. Crossing with *M. intermedia*, on the other hand, has strongly counteracted mutations in *M. tenuifolia*. As an instance of this the following cultivations may be mentioned.

Fruits from 5 plants of the type $b \times e$, which were found among the offspring of $b \times e$, were collected in 1927. The plants growing out of the seeds of each of these in the year 1929 were the following: (1) 8 b + 1 e + 18 $b \times e$, (2) 9 b + 11 e + 20 $b \times e$, (3) 23 b + 28 e + + 48 $b \times e$, (4) 18 b + 22 e + 35 $b \times e$, (5) 16 b + 11 e + 28 $b \times e$. In all, the offspring of these 5 $b \times e$ thus consisted of 74 b + 73 e + + 149 $b \times e$, but there was no case of a mutation of e into another biotype. In this respect the hybrid $b \times e$ differed clearly from $c \times e$ and $d \times e$.

It was a remarkable fact that the plants e through repeated cultivation as hybrids with b changed physiologically so as to lose more and more the ability to develop seed. Gradually they could become completely sterile. The anthers remained unopened, but this was not the cause of the sterility, for supply of pollen to the stigmas of the pistil could not prevent sterility. The cause of the sterility was evidently an inadequate supply of nourishment from those leaves whose preparation of nourishment had been weakened by decrease in the size of their laminae. This assumption is strengthened by the fact that fruits developed normally in cases where the shoots were provided with leaves with large laminae.

These physiologic changes in *M. tenuifolia* deserve to be illustrated by reference to some cultures of this biotype in which they have appeared. In the offspring of the hybrid $c \times e$ there appeared in the year 1913 one $b \times e$ through mutation. Fruits from one $b \times e$ were taken from the offspring of this hybrid for further cultivation. The 5 cultures grown in the year 1929 and referred to above belonged to the fifth generation produced in this way. The second of these cultivations, consisting of 9 b + 11 e + 20 $b \times e$, was chosen for further investigation. Of this cultivation the 11 e plants were the most sterile ones. Only in a few places on the stalks had an isolated, weakly developed fruit ripened. From one of these plants two fruits were obtained, and from their seeds there grew 10 e plants in the following year, which were all weakly developed. Fruits were taken from some of these for sowing later on, and the offspring obtained then was 13 plants of the type e. No mutation had taken place, but the plants were more strongly developed than those of the preceding generation. Fruits were collected only from one of these plants, and in the following year their seeds produced 25 e plants, which were very strongly developed. Fruits obtained from one single plant among these produced no fewer than 5 large cultures in 1935. Three of these, those sown last in the middle of the summer of

1935, consisted of: (1) 16 *e* + 2 *e* × ?, (2) 24 *e* + 2 *e* × *b* + 2 *e* × *c*, (3) 22 *e* + 1 *e* × *b* + 1 *e* × *c* + 1 *e* × *f* + 2 *c* × *a*. The plants in these three cultures became very strongly developed, having germinated and developed at the unusually high temperature occurring in the middle of the summer of this year. Two of the 5 cultures of *M. tenuifolia* had been sown earlier, and these resembled each other, but especially in two respects they differed from the three cultures sown later. They each consisted of 33 *e*, but none of the hybrids found in the three other cultures of this biotype appeared. Furthermore, the plants in these three cultures had much smaller leaves with smaller laciniae than those of the three characterized by very strongly developed plants. As the soil as well as the seeds were similar in all the five cultures, these differences must be due to the time of sowing being different. The two weak cultures had been sown 6 days earlier (June 12), and at this time the temperature was still unusually low this year. Germination had taken place in soil of a low temperature, and evidently the development then undergone by the sprouts influenced the subsequent growth. The low temperature probably also hindered the germinating and development of the vegetable substances that must have also been present in the seeds sown earlier, as these were collected from the same plant of *M. tenuifolia* as the seeds sown later.

Investigations concerning the appearance of new biotypes originating in the cultures from *M. oxyloba* (Fig. 1 *c*) have shown that the hereditary changes causing their appearance could lower as well as further their vital functions. The new biotypes *M. digitata* (Fig. 1 *d*) and *M. tenuifolia* (Fig. 1 *e*) were characterized by increased laciniation of the leaves. This modification reached its limit in the latter, the elaboration of food material by which was decreased in such a high degree that under certain circumstances no fruiting took place because of insufficient supply of nourishment. Hereditary changes in the opposite direction could restore the biotypes formed earlier and having better developed leaves. *M. tenuifolia*, when in a stage of more active production of biotypes with improved vital functions, could also give rise to *M. parviflora* (Fig. 1 *a*). But while in this stage it could also produce a new biotype, *M. rediviva* (Fig. 1 *f*), which was better organized than the other biotypes.

The hereditary changes giving rise to the aforementioned biotypes took place in the sex-cells, but in *M. tenuifolia* a sort of hereditary change could also take place in the vegetative cells. A brief account of this will be given in the following.

VEGETATIVE MUTATIONS.

The differences of leaf-structure peculiar to the 6 biotypes described on the foregoing pages were of such a kind as to be distributed among the offspring according to the law of monohybrid segregation. In one of these biotypes, *M. tenuifolia*, often in its hybrids with *M. digitata* and *M. oxyloba* as well, and always in its hybrids with *M. rediviva*,

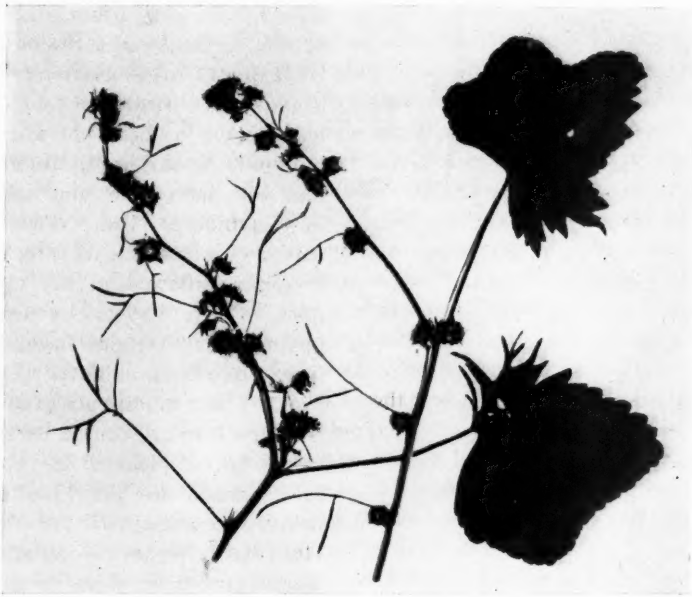


Fig. 3. Large leaf, caused by vegetative mutation, on each of the stalks of 2 plants of *M. tenuifolia*.

there appeared a transformation of the leaf-structure, which was of quite another kind than the leaf-structures characteristic of the different biotypes caused by mutation in the sex-cells. This made the investigations into the biotypes much more difficult. It was not made clear until after many years of research by cultivation that the forms produced in this way were highly different from the normal leaf-structure of the biotypes. The simplest form of the appearance of this discrepancy is illustrated in Figs. 3 and 4. In Fig. 3 are seen the upper parts of the stalks of 2 plants found in a culture of numerous plants of *M. tenui-*

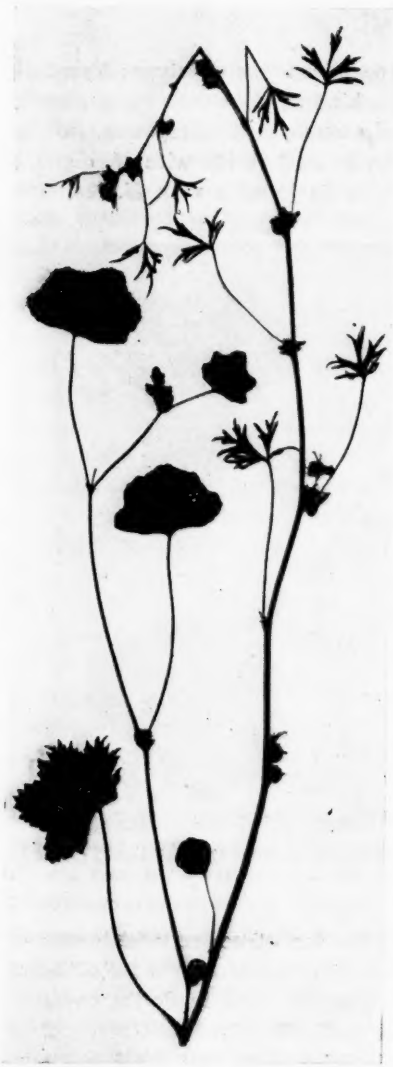


Fig. 4. A branch with undivided leaves has appeared from the fold of a slightly laciniated leaf on the stalk of a hybrid between *Malva tenuifolia* and *Malva oxyloba*.

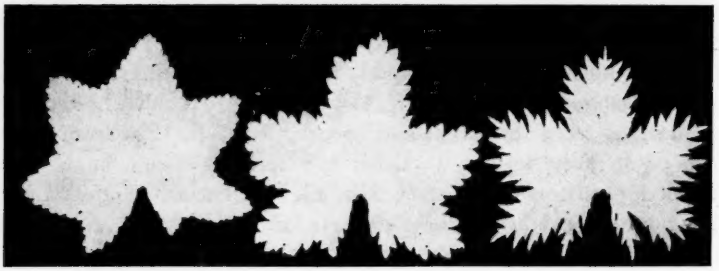
folia. On each of the stalks a leaf has appeared that has undergone, increasingly from one edge of the lamina to the other, a modification into the leaf-structure found in *M. rediviva*. In Fig. 4 is seen the upper part of the stalk of a hybrid between *M. tenuifolia* and *M. oxyloba*, one branch at the bottom having leaves resembling those found in *M. rediviva*. The branch starts from the fold of a leaf having a form not uncommon in *M. tenuifolia*. Entire branches with such leaves may appear in this biotype. Quite often the leaves characteristic of this biotype are followed in the upper part of the shoots by leaves having broad laciniae instead of thread-like ones, as those seen in Fig. 3. These modifications of the leaf-structure are caused by mutations taking place in the vegetative parts of the plant and thus being, according to DE VRIES (1901—03), vegetative mutations. These mutations were in many respects highly different from those taking place in the sex-cells. Hereditarily, a quality provoked by a vegetative mutation behaved quite differently from one caused by a mutation in the sex-cells and consisting of a certain modification of the gene determining the form of the leaves in the absence of disturbances caused by vegetative mutations. The most important of the numerous investig-

ations that were necessary in order to obtain a more detailed knowledge of these vegetative mutations will be mentioned here.

The peculiar *tenuifolia* biotype also appeared in the *Malva* cultures in Berlin-Dahlem described by F. A. LILIENFELD (1929). These cultures were derived from seeds that had been collected in 1920 from 2 small plants of *M. parviflora* in a garden at Alnarp in Scania in the neighbourhood of the cultures treated in the present work. Pollen could easily be transferred from these cultures to the 2 plants of *M. parviflora* by bees.

In 1914, a side-shoot, the leaves of which resembled those of *M. rediviva*, appeared on a plant of *M. tenuifolia*. The following year 10 plants grew out of its seeds, 4 of which resembled *M. rediviva* more or less. The leaves of the other plants were slightly divided at different degrees. In a few of them the leaves were almost as strongly divided as in *M. oxyloba*. Out of the 4 plants resembling *M. rediviva*, only 2 yielded a homogeneous offspring after sowing of the seeds of the 10 plants in 1917, the number of their offspring being 11 and 45, respectively. Subsequent generations of these also kept entirely homogeneous. The shape of their leaves is given in Fig. 5 as form *i*, which resembles that of the leaf *f* (in Fig. 1). Also later, side-shoots of *M. tenuifolia* or of one of its hybrids with *M. oxyloba* or *M. digitata* have yielded, by selection and repeated cultivation, offspring presenting the homogeneity characteristic of a biotype.

It is true that the vegetative mutation has been able to prevent the manifestation of the effects caused by the gene *e* in *M. tenuifolia* before the appearance of the mutation. It has not, however, been able to change the nature of this gene, as is evident from the following experiments, which show that a *M. tenuifolia* having a leaf-structure that has been modified by a vegetative mutation can be liberated from the quality caused by this mutation by crossing with *M. parviflora*. Its gene *e* has thus not been influenced by the vegetative mutation. In the experiments use was made of the form *i* mentioned above of *M. tenuifolia* in its ninth generation after its first appearance in 1924. Two plants out of a culture consisting of 31 plants of this form were fertilized by pollen from *M. parviflora* in 1932. In the following year, 10 and 7 plants, respectively, came from one fruit from each of these plants. They all resembled each other. The laciniation of their leaves resembled that of the leaf *j* (in Fig. 5). The crossing with *M. parviflora* had thus weakened the ability of the factor brought forth by the vegetative mutation to determine the form of the leaves. One of these *j*-like



i *j* *k*

Fig. 5. Three forms of *M. tenuifolia* brought forth by vegetative mutations.

hybrids $i \times a$ produced the following 30 plants in 1934: 7 a + 10 j + + 4 $a \times j$ + 1 $a \times k$ + 8 $a \times e$ (the forms of the leaves are represented by the same signs as those used in Figs. 1 and 5). The signs j and k approximately represent the forms under which *M. tenuifolia* is as yet hidden. The presence of 8 $a \times e$ implies a complete annihilation of the effect of the vegetative mutation in this hybrid union. One of the hybrids $a \times j$ produced the following offspring in 1935: 5 a + 7 e + + 1 $a \times j$ + 12 $a \times e$. Four out of these 7 e had leaves in the upper part of the stalks with broader laciniae than those of the leaves lower down. This phenomenon is also rather common in cultures of pure *M. tenuifolia*. The result of these experiments was that the form-determining factor provoked by a vegetative mutation gradually lost its importance when influenced by the gene determining the leaf-structure of *M. parviflora*. It has been pointed out above that *M. rediviva* differed from *M. parviflora* in several respects. These biotypes were the opposites of each other as to their effect on the vegetative mutation. This is made clear by the following observations, which have also been mentioned earlier in this paper.

The leaves of a hybrid between *M. tenuifolia* and *M. rediviva* were of the form seen in Fig. 2 *h*. This hybrid had leaves of the same form as in the offspring of the plant mentioned, but here *M. tenuifolia* was generally not represented in its normal form, its leaves being influenced in various degrees by a vegetative mutation. Its leaves were of forms between *e* (in Fig. 1) and *j* or *k* (in Fig. 5). Sometimes, however, an isolated instance of a pure *M. tenuifolia* occurred in the offspring of the hybrid mentioned. In the offspring of other hybrids with *M. tenuifolia*, the latter always appeared in its normal form. It is thus evident

that *M. rediviva* was favourable to the vegetative mutation. But the vegetative mutation could also, as was mentioned before, make its presence felt in cultures of pure *M. tenuifolia* and of its hybrids with *M. digitata* and *M. oxyloba*. Three of the numerous forms that could be brought forth by vegetative mutation are shown in Fig. 5 by the leaves *i*, *j* and *k*. The first of these had become, by selection, altogether constant in multiplication. This also applies to the form *j* following next. It had, as well as the former, appeared long before the year 1927, but the third form, with the leaf *k*, originated from a culture of the year 1930, where it was the only one of its kind among 12 other plants having leaves that resembled *j* (in Fig. 5). In the following year, this plant with leaves of the form *k* produced 19 *k* and 3 plants with leaves of a form approaching that of normal *M. tenuifolia*. In the next culture, one of these 19 plants of the type *k* produced 15 *k* besides 4 plants of normal *M. tenuifolia* and 4 plants having a leaf-structure closely akin to that of normal *M. tenuifolia*. The offspring of one of these 15 *k* consisted of 31 *k* and 4 plants with other types of leaf-structure. One of these was closely akin to *M. tenuifolia*. The leaf-structure of the three others bore a certain resemblance to *h* (in Fig. 2) and *j* (in Fig. 5). Only their descendants in a subsequent culture could have revealed their origin. The results of the 3 cultures of the form *k* following upon each other do, however, clearly show that the factor owing its origin to a vegetative mutation was weakened in such a high degree that it had no influence at all on the leaf-structure of some of the plants, and thus the gene *e* could determine the leaf-structure alone.

SUMMARY.

In a culture of *M. oxyloba*, which is closely related to *M. parviflora*, there appeared by and by new biotypes differing from each other as to their leaf-structure. The hybrids between them were monohybrids. In the absence of vegetative mutations influencing the leaf-structure, the latter was accordingly determined by a gene that in each biotype owed its origin to a mutation from one and the same gene. In the majority of the new biotypes the leaves were ever more strongly laciniated. In the biotype having the most strongly laciniated leaves, *viz.* the one given the name *M. tenuifolia*, the fruiting was sometimes poor or altogether absent because of insufficient supply of nourishment from the leaves. Biotypes with laciniated leaves could often reproduce the earlier types with less divided leaves. When *M. tenuifolia* was in a

modification favouring the production of biotypes with better leaf-structure than its own, *M. parviflora* could also appear in its offspring. Yet another biotype could then appear, which differed from all the biotypes previously formed. It was the one appearing last in the series of new biotypes and was given the name *M. rediviva*. It bore a certain resemblance to *M. parviflora*, but the leaves were less divided. It also surpassed *M. parviflora* in having a stronger influence on the leaf-structure of its hybrid with a biotype possessing laciniated leaves, whereas the latter had a stronger influence on the leaf-structure of its hybrid with *M. parviflora*.

In *M. tenuifolia*, as well as in its hybrids with closely related laciniated biotypes, vegetative mutations could bring forth leaves that in some cases resembled those of *M. rediviva* and in some cases were in various degrees more weakly laciniated than those of *M. tenuifolia*. This change of form could appear in individual leaves on the shoots of a *M. tenuifolia* whose other leaves were of the normal form. The upper part of its shoots often had only larger leaves with broader laciniae. There could also appear side-shoots bearing in their entirety leaves of the form characteristic of *M. rediviva*. One of these shoots fruited abundantly. Numerous plants grew out of its seeds, but they were of varying leaf-structure. Only a few of the plants had leaves resembling those of the mother-shoot. By selection and continued propagation they appeared ever more abundantly in the offspring, and after a few generations the new form was altogether constant, although only when it seeded through fertilization by its own pollen, whereas crossing with *M. parviflora* and continued propagation of the hybrids thus formed counteracted the effects of the vegetative mutation, bringing forth *M. tenuifolia* of normal leaf-structure. In this biotype the gene had at its first appearance thus remained unaffected by the vegetative mutation.

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ABSTRACTS — KURZE MITTEILUNGEN

K. A. JENSEN, G. KÖLMARK and M. WESTERGAARD: Back-mutations in *Neurospora crassa* induced by diazomethane.

In the mould *Neurospora crassa* a number of so-called biochemical mutants have been induced in which the capacity to carry out a specific enzymatic reaction, necessary for the normal growth of wild type strains, is blocked. Hence the mutant will only grow if the specific substance normally synthesized by the enzymatic reaction is added to the culture medium (BEADLE and TATUM, 1941). In some cases such biochemically deficient strains may revert after some time to wild type growth again, *e. g.* the will grow on the »minimal medium (see BEADLE and TATUM, 1945) without the specific substance added. In some cases these reversions are due to non-genetic adaptations, in other strains, however, the reversion to wild type growth is due to a back-mutation in the gene controlling the biochemical reaction (see GILES and LEDERBERG, 1948; KÖLMARK and WESTERGAARD, 1949).

Such back-mutations offer a useful tool for studying the spontaneous and induced mutability of specific genes, since it is possible, by using a screening technique, to treat and investigate a very large number of nuclei, of which only those which have back-mutated will be scored (see GILES and LEDERBERG, 1948; KÖLMARK and WESTERGAARD, 1949, for details).

For several years we have been using an adenineless strain, combined with a gene for colonial growth, as a tool for studying the back-mutation rate of the adenineless gene to wild type. It has been found that the back-mutation rate can be increased considerably by treatment with X-rays, ultra-violet light or nitrogen mustard (KÖLMARK and WESTERGAARD, 1949). A number of other compounds have also been tested, which have been claimed by other authors to have a mutagenic effect when tested by the *Drosophila* ClB test or by the *Allium* test. These include phenol (mutagenic effect claimed on basis of autosomal lethal and visible mutations in *Drosophila*, II chromosome; see HADORN, ROSIN and BERTANI, 1949), phenol derivatives like pyrogallol, hydroquinone and pyrocatechol (mutagenic effect demonstrated by means of *Allium* test; see LEVAN and TJIO, 1948) and urethane (mutagenic effect demonstrated on *Oenothera* by OEHLKERS, 1946, on *Drosophila* by ClB test by VOGT, 1948). *All these chemicals, however, failed to increase the back-mutation rate of the adenineless gene significantly.* A detailed report on these and other negative experiments with chemicals will be published later.

It was therefore decided to search for other compounds with a chemical reactivity similar to that of nitrogen mustard, and to examine such chemicals for mutagenic activity by means of the back-mutation test.

Nitrogen mustard is characterized by some very active chlorine atoms; in neutral or alkaline solutions it is almost instantaneously transformed into a cyclic onium cation with liberation of Cl^-. If the mutagenic effect of nitrogen mustard is in some way or other linked with the liberation of energy during its chemical reaction, one might expect a number of other compounds,

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with a similar high reactivity, to have a similar biological effect. Very few such compounds have so far been tested for mutagenity, probably because most of them immediately react with water, and the chance of bringing them in contact with the cell nucleus has been regarded negligible.

However, we have found it worth while to test a number of such very reactive compounds systematically by means of the back-mutation test, and among those so far tested one, *viz.* diazomethane, CH_2N_2, has been found to be rather strongly mutagenic.

Diazomethane was applied to the *Neurospora* spores by adding 0,5 cc of a freshly prepared diazomethane solution of known concentration in dry ether to 8 cc of a spore suspension in sterile water. As soon as the ether solution was added, the test-tube was shaken violently. Nitrogen was immediately evolved. The mixture was then agitated for 40 minutes in a shaker machine. The reaction was stopped by centrifuging the spores and washing them with two changes in sterile water. The general procedure is the same as that described by KÖLMARK and WESTERGAARD, 1949.

The results of two experiments are shown in Table 1. Since the conidia treated are multinucleate, the figures indicate the mutation rate per million spores, not per million nuclei (see KÖLMARK and WESTERGAARD, 1949). The molar concentrations given in the table correspond to the concentrations of diazomethane in dry ether. The toxicity of diazomethane appears to be less

TABLE 1.

Treatment	Numb. of spores	% survived	Numb. of mutations	Mutations per 10^6 initial spores	Mutations per 10^6 survived
Control	44 $\times 10^6$	100	2	0,05	0,05
Diazomethane 0,25 mol. 4° C.	9,5 $\times 10^6$	22	18	0,41	1,89
Diazomethane 0,25 mol. 25° C.	8,3 $\times 10^6$	19	28	0,64	3,37
Ultra-violet light, 60 sec.[1]	20,0 $\times 10^6$	45	130	2,95	6,5
Control: 0,5 cc of dry ether to 8 cc of spore solution in 40 minutes	30 $\times 10^6$	68	1	0,02	0,03
Control	47 $\times 10^6$	100	1	0,02	0,02
Diazomethane ca. 1 mol. 25° C.	3,5 $\times 10^6$	7	20	0,43	5,7
Diazomethane 0,1 mol. 25° C.	27,7 $\times 10^6$	63	17	0,36	0,61
Nitrogen mustard 1/200 mol. pH 9,5. 30 minutes. 25° C.	0,71 $\times 10^6$	0,2	14	0,30	17,9
Urethane 1 mol. 120 min.	6,0 $\times 10^6$	13	0	0	0

[1] From a Hanovia lamp, yielding about 13.200 erg per square centimetre per second.

than that of nitrogen mustard, probably due to the fact that most of the diazomethane is destroyed by water, saving only a small amount to react within the cell. The urethane experiment included in the table is one of many negative experiments with varying concentration and exposure time; similar negative experiments have been carried out with phenols.

The demonstration of the mutagenic effect of diazomethane is of considerable interest, especially in the light of the negative results obtained with phenols and urethane.

Diazomethane is known to transform hydrogen atoms of sufficient acidity into methyl groups according to the following reaction:

$$—H + CH_2N_2 \rightarrow —CH_3 + N_2.$$

It reacts, for instance, with carboxylic acids to form esters, with phenols to form anisols and with purines to form N-methyl purines. In all these reactions nitrogen is evolved with liberation of energy. It reacts with water to form methylalcohol:

$$H_2O + CH_2N_2 \rightarrow CH_3OH + N_2.$$

In the case of nitrogen mustard it has been demonstrated that it reacts with the nucleic acids of the chromosomes. A special strong affinity has been found for the transformer principle of *Pneumococcus* (HERRIOTT, 1948). It seems probable that diazomethane likewise reacts with the nucleic acid molecules, although no experimental data are yet available.

The mutagenic effect of diazomethane may be explained by several hypotheses. One is, that the back-mutation is due to a direct methylation of the gene molecule itself. This, however, is not very likely in view of the fact that the same type of mutation, *e. g.* the back-mutation in the adenine locus, can also be induced by means of X-rays, ultra-violet light and by nitrogen mustard, none of which have the same specific chemical effect. It seems also unlikely that a back-mutation, *e. g.* a transformation of a mutated gene into its original form, should be due to a methylation. Another hypothesis in explanation of the mutagenic effect of diazomethane seems much more likely, *viz. that the capacity of the gene molecule to mutate, in the present case to back-mutate, is a latent possibility in the gene molecule itself, which can be released by the transfer of energy liberated from the chemical reaction.* This energy transfer would then promote an intramolecular rearrangement to take place, for instance, a cis-trans rearrangement or the like. In that case the effect of diazomethane would also be of the »treffer» type, as already suggested for nitrogen mustard by AUERBACH (1949) and for the mutations induced by radiation by TIMOFÉEFF-RESSOVSKY, ZIMMER and DELBRÜCK (1936; see also LEA, 1946). Hence the primary effect of the chemical mutagens would be, as stated by AUERBACH (1949, p. 139): ». . . a transfer of energy to a localized spot of the chromosome».

If this is the correct explanation, and it may, at least for the time being, serve as a good working hypothesis, it should not be expected that comparatively only slightly reactive substances like phenols, urethane, azo-compounds (see DEMEREC, 1949) and possibly also the carcinogens should have a

mutagenic effect, and, in fact, *they have failed to yield any significant results in our experiments*. It should be remembered, however, that the mutagenic effect of these compounds has been demonstrated by different methods, *e. g.* by the ClB test or by the *Allium* test. Both these tests are first and foremost tests for chromosome aberrations and lethals, whereas the back-mutation test might be regarded as a more specific test for gene mutations, *e. g.* reversible intramolecular rearrangements. It seems likely that a number of highly toxic compounds may be able, in one way or another, to induce chromosome breakages, or simply, by irreversibly »killing» the genes, induce lethal mutations, whereas the same compounds would not be able to induce the reversible intramolecular changes which are probably scored by the back-mutation test.

On the other hand, it should be pointed out that, according to the hit theory, a number of other chemicals might be expected to be mutagenic because of their strong reactivity. Especially it would be very interesting to investigate the activity of free radicals, because it is not unlikely that free radicals, produced during cell metabolism, may produce mutations if reacting with or near the gene molecules. In that case the mechanism which produces the spontaneous mutations may be viewed from a new angle.

Summary. — Back-mutations of the adenineless gene to wild type have been induced in an adenineless strain of *Neurospora* by means of diazomethane, CH_2N_2, whereas phenols and urethane have failed to yield any significant results. The implications of this have been discussed in relation to the possible mechanism of chemically induced mutations.

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GUNNAR ÖSTERGREN: A survey of factors working at mitosis.

A study of the movements and equilibria of the chromosomes at mitosis has revealed a number of influences responsible for these phenomena. The results may be said to be of two kinds, *viz.* firstly an abstraction of empirical tendencies of behaviour, and secondly a theoretical interpretation of these tendencies. Some of the results have already been presented in a number of publications by the present writer and co-workers (ÖSTERGREN, 1945 a and b, 1947, 1949 a, b; ÖSTERGREN and PRAKKEN, 1946; BÖÖK, 1945; PRAKKEN, 1948) and other results are now being worked out for publication in a series of papers.

There are many factors influencing the effects under study, and it seems useful to give a survey of these factors before entering into the details of the analysis. In the coming papers there will be made repeated reference to the present survey. Only the empirical factors of behaviour will be regarded here. Their theoretical interpretation is given elsewhere (ÖSTERGREN, 1949 b and unpublished). The evidence for the present factors is partly given in the publications cited above and will partly appear in the coming papers.

The subject under study is the *position*, *movement* and *shape* of chromosomes and chromosome configurations on the spindle. Excluded from the present study are: the mechanism of the meiotic pairing, that of the somatic pairing, the formation of chiasmata, the problems of chromosome spiralization, heterochromatin questions and questions of chromosome metabolism.

The nature of some of the present factors is quite obvious, such as, *e. g.*, the *relic factors* or the *elasticity* of the chromosomes; in such cases the nomenclature is no problem. In the case of certain other factors, some of which are very important for the explanation of the chromosome behaviour, their ultimate nature is, however, very obscure. For such cases I have found it suitable to give them quite non-committal names by denoting them simply with Greek letters.

I. The factors of behaviour.

A. *Spindle factors.*

1. The *α-factor.* An attraction of centromeres to spindle poles by forces increasing in strength with increasing distance from the pole. This attraction acts only towards that pole towards which the centromere is oriented. This is the factor causing the prometaphase movement to the equator and the anaphase movement to the poles.

2. The *β-factor.* A mutual influence of chromosomes or chromosome con-

figurations in their movements parallel to the spindle axis of such type that they tend to follow their neighbours. This is an important factor modifying the result of the α-factor.

3. The *γ-factor.* There is a tendency of the spindle to extrude bodies out of it, acting, *e. g.*, on chromosome arms and nucleoli. I have the notion that there may be two components in this factor: γ_1, which is probably simply the surface tension of the spindle body, and γ_2, which may be a more specific factor due to more complicated organizational features of the spindle and manifested especially (or exclusively?) in organisms with centrosomes, where it is also recognizable as a repulsion of the chromosome arms from the spindle poles.

4. The *δ-factor.* The tendency of the spindle to arrange rod-shaped bodies in parallel to the spindle fibres.

5. The *ε-factor.* There may be a kind of crowding effect between the chromosomal spindle fibre bundles contributing to the spreading of the centromeres on the metaphase plate. The existence of this factor is not definitely clear, and this interpretation of it as a crowding of fibre bundles is admittedly somewhat hypothetical.

6. The *anaphase elongation of the spindle.* The »Stemmkörperwirkung». In some cases (especially in animals) there is a considerable elongation of the spindle during anaphase (*cf.*, *e. g.*, RIS, 1949). This naturally contributes to increasing the distance between the separating chromosomes. In many cases, however, this effect is absent or negligible.

B. *Chromosome factors.*

7. The *elasticity* of the chromosomes, which tends to prevent them from deviating from a definite »ideal shape», which characterizes them when they are free from external forces.

8. The *plasticity* of the chromosomes. The changes to which the chromosomes are subjected by the forces of nuclear division are not all elastically reversible, but there seems to be a certain degree of plastic deformation.

9. The *size* and *shape* of the chromosomes; the shape factor is the »ideal shape» just referred to. Here should also be mentioned the association of chromosomes in pairs or larger configurations at the first division of meiosis, a feature which conditions the process of co-orientation.

10. The *centromere factor.* The presence in a chromosome of one centromere, two or more, or no centromere at all (acentric fragments), or the presence of a diffuse centromere instead of a localized one. Here belong also the changes in centromere constitution during the progress of the cycles of mitosis and meiosis (its state of polarization).

11. Body repulsion. There may possibly be a slight repulsion (due to surface charge?) between chromosome bodies, working at very short distances and reducing the instances of direct contact. At least, it seems difficult to exclude the possibility of such an effect.

12. The factors causing *secondary pairing* (at meiosis) and *somatic pairing* (at mitosis). In many organisms there is no definite evidence for the existence of these effects, in others there are quite clearly such effects, *e. g.* somatic

pairing in Diptera. The analysis of these more specific effects is outside the scope of the present series of works, but they must naturally be mentioned in a survey like the present one.

C. *General medium factors.*

13. Viscosity of the surrounding medium. The viscosity of the medium offers resistance to the chromosome movements, either the moving chromosome parts are lying in the spindle or in the surrounding cytoplasm.

14. Cytoplasmic streamings. Chromosome parts lying in the cytoplasm are naturally influenced by cytoplasmic currents.

D. *Relic factors.*

15. Relics of movement. The arrangement of chromosome arms is often a relic from a previous movement. Thus, at the end of anaphase the chromosomes continue to lie with their centromeres in a polar group and with the arms pointing towards the previous equator.

16. Relic spiral. Some of the curving of the chromosomes during prometaphase and early metaphase is most probably the last remnants of the relic spirals usually observed so easily in prophase.

II. Additional remarks.

One further factor that might be mentioned is the *inertia* due to the *mass* of the chromosomes, a factor that theoretically should play some rôle in accelerations and retardations of their movements; the chromosome movements are, however, very slow, and there is no practical evidence that inertia plays a noticeable rôle. Besides the above factors which act on the chromosomes when they are on the spindle, there may be other factors influencing them inside prophase nuclei, such as attachment to the nuclear membrane, *etc.* These are, however, outside the scope of the present paper.

One factor that many readers may have missed in the above description is the localized centromere repulsion suggested by DARLINGTON (*e. g.* 1937) and others to act between centromeres. The reason why this factor has not been taken up here is simply that there is no evidence that clearly demonstrates its existence, and I do not want to complicate the interpretations by making such unnecessary assumptions. I am, however, well aware that a more detailed analysis of the chromosome behaviour in the future may force us to introduce an effect of this kind in our interpretations.

The above description may be supplemented by the remark that there seem to be, as far as can be judged at present, only two kinds of reaction known of bodies towards the spindle; they may be called *centromere behaviour* and *neutral body behaviour.* The first kind of behaviour is characterized by the α-, β-, and ε-factors mentioned above, and the second kind by the γ- and δ-factors. Centromere behaviour is a specific type of behaviour characterizing centromeres, and as an abnormality also special chromosome-ends in certain inbred strains of rye (called T-ends; *cf.* ÖSTERGREN and PRAKKEN, 1946) and some chromosome parts in maize plants having an abnormal chromosome 10

(RHOADES and VILKOMERSON, 1942). The other kind of behaviour is most probably a quite unspecific type that would be manifested by any foreign body that has been included in the spindle.

The spindle elongation during anaphase (in cases where it exists) may also be regarded as a quite unspecific factor of movement; obviously any bodies included in an elongating spindle will thereby get an increased distance from one another.

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